NOVEL SYNTHETIC ANTIMICROBIAL PEPTIDES WITH *IN-VIVO* AND *IN-VITRO* ACTIVITY AGAINST *STREPTOCOCCUS PNEUMONIAE*

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ABSTRACT

Streptococcus pneumoniae is a major human bacterial pathogen causing life threatening diseases such as meningitis, bacteremia, and pneumonia. Younger children are heavily affected by pneumococcal infections. Pneumococcal conjugate vaccines (PCVs) are serotype-specific and require data on local serotype distribution to predict the coverage of PCVs. Moreover, the escalating incidence of antibiotic resistance *S. pneumoniae* has prompted the development of novel antimicrobial agents. Antimicrobial peptides (AMPs) have been increasingly recognized as the new generation antibiotics due to their potent antimicrobial activity. In this study, we sought to design novel synthetic antimicrobial peptides against *S. pneumoniae* and determine the *in vitro* and *in vivo* activities of the peptides.

A collection of 151 pneumococcal clinical isolates from University of Malaya Medical Centre, Kuala Lumpur were serotyped using multiplex PCR and the penicillin susceptibility was determined using agar dilution method. Biocomputational tools were employed to design the peptides based on the publicly available AMPs. The designed AMPs were tested for minimum inhibitory concentration, bacterial killing kinetics, and synergism activity. Broad spectrum antibacterial activity against eight common bacterial pathogens was also determined. Morphological changes of pneumococcal cells were observed using transmission electron microscopy. The designed AMPs were also assessed for hemolytic and cell cytotoxicity. *In vivo* therapeutic efficacy and acute toxicity of the peptides were assessed using an in-house mouse model.

Serotypes 19F and 23F were the two prevailing serotypes among Malaysian population with serotype 19F significantly associated with penicillin resistance. Penicillin-nonsusceptible *S. pneumoniae* constituted half (50.3%) of the total isolates. and as high as 87.5% of penicillin-resistant *S. pneumoniae* (PRSP) were PCV7-vaccine serotypes. From the peptide designs, antimicrobial testing showed that the five hybrid

peptides (DM1-5) exhibited strong antipneumococcal activity irrespective of penicillin susceptibility of the isolates. These isolates included the highly prevalent serotype 19F. The pneumococcal killing rates were higher than penicillin by 38 – 64%, 54 – 76%, and 30 – 57% for penicillin-resistant, intermediate, and susceptible isolates at 30 min posttreatment, respectively. The DMs and other peptides produced synergism in combination with penicillin against *S. pneumoniae* and displayed broad spectrum antibacterial activities. The DMs induced overwhelming cellular damages leading to cell death. Besides, DMs exhibited low hemolytic activity (HC_{50} >250 μg/ml) with varying degrees of cell cytotoxicity against NL20 and A549 cell lines. Notably, DM3 (40 mg/kg) given via intraperitoneal route at 12 hrs interval for three dosing regimens protected 50% of mice from lethal systemic infection by a PRSP strain. Interestingly, combination therapy using low doses (10mg/kg and 20mg/kg) DM3 and penicillin showed therapeutic synergism with 20-50% higher survival rates than the sum of the standalone treatments. Complete protection (100%) was achieved with DM3 (20 mg/kg) – penicillin (20 mg/kg) combination.

Pneumococcal infections continue to affect humans and antibiotic-resistant *S. pneumoniae* would further complicate the treatment outcomes. The current study demonstrated that AMPs represent the promising new generation antibiotics as standalone therapeutics or in combination with conventional antibiotics. Novel designed AMPs candidates can be designed following careful designing approaches to generate peptides with high therapeutic potential to be further developed into clinically useful antibiotics.

ABSTRAK

Streptococcus pneumoniae merupakan sejenis patogen bakteria yang boleh menyebabkan pelbagai jenis penyakit yang mengancam nyawa manusia seperti meningitis, bakteremia, dan pneumonia. Kanak-kanak merupakan golongan utama yang sering diancam oleh jangkitan pneumokokal. Keberkesanan "pneumococcal-conjugate vaccine (PCV)" adalah spesifik kepada jenis serotype dan data mengenai taburan serotype tempatan amat diperlukan supaya liputan PCVs dapat dianggarkan. Selain itu, isu-isu peningkatan rintangan antibiotik *S. pneumoniae* telah meningkatkan permintaan terhadap antibiotik baru. Peptide antimikrobial (AMPs) mempunyai aktiviti antimikrob yang tinggi dan telah kian diiktiraf sebagai antibiotik generasi baru. Dalam kajian ini, kami telah mereka peptida sintetik terhadap *S. pneumoniae* dan menentukan aktiviti-aktiviti *in vitro* dan *in vivo* peptida tersebut.

Sebanyak 151 isolat pneumokokal klinikal dari Pusat Perubatan Universiti Malaya, Kuala Lumpur telah diserotypekan menggunakan PCR multipleks dan tahap rintangan penisilin ditentukan dengan kaedah pencairan agar. Teknik biokomputer digunakan untuk mereka peptida baru berdasarkan peptida yang sedia ada. Seterusnya, peptida sintetik tersebut diuji untuk menentukan "minimum inhibitory concentration (MIC)", kinetik penghapusan bakteria, dan aktiviti sinergi. Peptida sintetik tersebut juga diuji untuk keberkesanan antibakteria specktrum luas terhadap lapan bakteria patogen. Perubahan morfologi sel pneumokokal telah divisualisasikan dengan menggunakan mikroskop elektron transmisi (TEM). Selain itu, tahap hemolitik dan ketoksikan peptida sintetik tersebut terhadap sel-sel manusia turut ditentukan. Keberkesanan terapeutik dan ketoksikan *in vivo* telah dinilai menggunakan model tetikus.

Serotype 19F dan 23F merupakan dua jenis serotype lazim di kalangan penduduk Malaysia. Serotype 19F adalah dikaitkan dengan rintangan penisilin. Separuh daripada jumlah isolat merupakan *S. pneumoniae* yang toleran kepada penisilin (50.3%).

Sebanyak 87.5% *S. pneumoniae* yang rintang penisilin (PRSP) merupakan serotype yang terkandung dalam vaksin PCV7. Ujian antimikrobial menunjukkan bahawa aktiviti DAMP7 adalah sederhana manakala lima peptida hibrid (DM1-5) mempamerkan aktiviti antipneumococcal yang kuat terhadap semua isolat pneumokokal tanpa bergantung kepada rintangan penisilin isolat termasuk serotype 19F. Kadar penghapusan pneumokokal adalah lebih tinggi daripada penisilin dan mencapai 38 - 64%, 54 - 76%, dan 30 - 57% bagi isolat resistan, sederhana, dan sensitif penisilin masing-masing selepas dirawat untuk 30 min. DAMP7 dan DMs dapat menghasilkan kesan sinergi terhadap *S. pneumoniae* semasa dikombinasikan bersama penisilin dan juga mempunyai aktiviti antibakteria spektrum luas. DMs juga menyebabkan kerosakan dinding dan membran sel yang akhirnya membawa kepada kematian sel. Peptida hibrid tersebut menunjukkan aktiviti hemolitik yang rendah (HC_{50} > 250 μg/ml) dan tahap ketoksikan yang berlainan terhadap sel-sel NL20 dan A549. Rawatan DM3 (40 mg/kg) secara intraperitoneal pada setiap selangan 12 jam untuk tiga regim dapat menyembuhkan 50% tetikus daripada jangkitan maut sistemik oleh PRSP. Terapi combinasi dengan dos rendah DM3 dan penicillin menunjukkan sinergi teraputik di mana kadar pelindungan sebanyak 20 – 50% lebih tinggi daripada rawatan tunggal dicatatkan. Perlindungan menyeluruh (100%) dapat dicapai dengan kombinasi DM3 (20 mg/kg) – penicillin (20 mg/kg).

Jangkitan pneumokokal akan terus mengancam manusia dan dijangka akan merumitkan lagi rawatan untuk penyakit-penyakit yang melibatkan *S. pneumoniae* rintang antibiotik. Kajian ini menunjukkan bahawa AMPs merupakan calon antibiotik generasi baru yang berpotensi untuk rawatan secara tunggal ataupun diformulasikan dengan antibiotik konvensional. Penyelidikan secara systematik dan teliti dapat membantu menghasilkan AMPs yang mempunyai potensi terapeutik yang tinggi untuk kegunaan klinikal.

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^{o}C	Degree Celcius
%	percent
µg	microgram
µl	microliter
µM	micromolar
µm	micromole
µmol	micromole
A	alpha
β	beta
fl	femtoliter
U	unit
V	volt
v/v	volume/volume
bp	base pair
G	gauge
g	gram
hrs	hours
kDA	kilodalton
kg	kilogram
l	liter
mg	milligram
min	minute
ml	milliliter
mmol	millimole
mM	millimolar
mm	millimeter
nm	nanometer
s	second
ALP	alkaline phosphatase
ALT	alanine transaminase
AMPs	antimicrobial peptides
APD	Antimicrobial Peptide Database
ARI	acute respiratory infection
AST	aspartate aminotransferase
BAL	bronchoalveolar lavage
BHI	brain heart infusion broth
CAMHB	cationically-adjusted Mueller Hinton Broth
CAP	community acquired pneumonia
CbpA	choline-binding protein A
CBPs	choline-binding proteins
CCI	charge compatibility index
CFU	colony forming units
CLSI	Clinical and Laboratory Standard Institute
CPS	capsular polysaccharide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
CSF	cerebrospinal fluid
EDTA	ethylene diamine tetraacetic acid

FBS	fetal bovine serum
FIC	fractional inhibitory concentration
GC	guanine-cytosine
GRAVY	grand average of hydropathy
Hb	hemoglobin
HCI	hydrophopathy compatibility index
HIV	Human Immunodeficiency Virus
HR	hydrophobic ratio
IN	intranasal
IP	intraperitoneal
IPD	invasive pneumococcal disease
IT	intrathoracic
LDH	lactate dehydrogenase
LHB	laked horse blood
LPS	lipopolysaccharide
LTAs	lipoteichoic acids
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MDR	multidrug resistance
MIC	minimum inhibitory concentration
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MRSA	methicillin-resistant *S. aureus*
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-. (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]
NA	nutrient agar
NetC	net charge
NP	nasopharyngeal
NVTs	nonvaccine serotypes
OD	optical density
OsO_4	osmium tetroxide
PBS	phosphate buffered saline
PBPs	penicillin-binding proteins
PCR	polymerase chain reaction
pCV	packed cell volume
PCV	pneumococcal conjugate vaccine
PCV7	7-valent pneumococcal conjugate vaccine
PCV10	10-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PDB	Protein DataBank
PEN	penicillin
PF	prophenins
PGLa	peptidyl-glyclyleucine-carboxyamide
PISP	penicillin-intermediate *S. pneumoniae*
PNSP	Penicillin-nonsusceptible *S. pneumoniae*
PPV23	23-valent pneumococcal polysaccharide vaccine
PRSP	penicillin-resistant *S. pneumoniae*
PSSP	penicillin-susceptible *S. pneumoniae*
PSCs	Possible structural combinations
QRDRs	quinolone resistance-determining regions
RBC	red blood cells
RFLP	restriction fragment length polymorphism

RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	rotation per minute
SC	subcutaneous
SD	standard deviation
SpsA	secretory pneumococcal surface protein A
TAs	teichoic acids
TAE	Tris-acetic EDTA
TEM	transmission electron microscopy
THV	total hydrophobic value
tRNA	transfer ribonucleic acid
UK	United Kingdom
UMMC	University Malaya Medical Centre
US	United States
VLSU	Veterinary Laboratory Service Unit
VPDs	vaccine-preventable deaths
VTs	vaccine serotypes
WBC	white blood cells
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1. Overview of *Streptococcus pneumoniae*

Streptococcus pneumoniae is a facultatively anaerobic, gram-positive lanceolate-shaped cocci which is commonly present as normal flora in the human upper respiratory tract (Gray *et al.*, 1980). The size of an individual cell ranges from 0.5 - 1.25 μm in diameter and frequently appears as a single cell, paired (diplococci), or short-chain form under the microscope. *S. pneumoniae* has no fimbriae and no fibrils (Tuomanen *et al.*, 1995). It produces hydrogen peroxide which oxidizes hemoglobin to methemoglobin. This gives the distinctive greenish-brown appearance under the growth of pneumococcal colony on blood agar and is termed α-hemolytic. Besides this, *S. pneumoniae* is catalase-negative, optochin (ethylhydrocupreine hydrochloride) susceptible, and bile (sodium deoxycholate) soluble. The optochin and bile solubility tests are common biochemical tests used to differentiate pneumococcus from the closely-related α-hemolytic viridans streptococci such as *Streptococcus mutans, Streptococcus mitis,* and *Streptococcus sanguinis. S. pneumoniae* undergoes autolysis after 12 - 16 hrs of incubation and this process can be enhanced in the presence of surface active agents such as sodium deoxycholate which inactivates the cell wall autolysin inhibitors.

S. pneumoniae is a naturally transformable microorganism. The presence of as much as 40 open reading frames in the R6 strain, which is one of the most widely studied pneumococcal strains certainly reflects how much this bacteria is readily transformable by the uptake of DNA (Hoskins *et al.,* 2001). Other features of the R6 strain include genome size of 2,038,615 bp, GC content of 40%, and 2043 protein coding regions. Transformation can create recombinant strains with enhanced survival fitness, disease-causing ability, and tolerability to antibiotics (Chalkley & Koornhof, 1990; Zhang *et al.,* 2005). Being part of the complex microbiota especially in the respiratory tract, *S. pneumoniae* is constantly interacting with various other bacterial

species. This includes co-colonizing pneumococcal strains and the closely-related streptococcal species. At the same time, *S. pneumoniae* is exposed to exogenous DNA released from the dead/lysed pneumococcal streptococcal cells present in microenvironments (Steinmoen *et al.*, 2002). Naturally competent pneumococci are able to pick up these exogenous DNA to be incorporated into its own genome and subsequently to have the recombinant genes expressed (Steinmoen *et al.*, 2002; Steinmoen *et al.*, 2003). However, upon acquisition of genes encoding the altered penicillin-binding proteins (PBPs), pneumococci can be transformed to express the recombined PBPs with lower affinity to ß-lactam antibiotics resulting in β-lactam resistance in the transformed pneumococcal cells (Dowson *et al.*, 1994).

1.2. Pneumococcal virulence factors

1.2.1. Pneumolysin

The processes of colonization and disease manifestations involve the cascades of coordinated switching and expression of an armamentarium of virulence factors to facilitate pneumococcal invasion from upper to lower respiratory tract (Kadioglu *et al.*, 2008). It is when *S. pneumoniae* spread to the respiratory lining epithelium at the site of infection do they express the disease-causing potential (Catterall, 1999). The complex interplay between pneumococcal virulence factors and host immune factors is crucial to prevent elimination by the host immune defense mechanisms and ensures efficient adaptation of pneumococci to the drastic changes in the microenvironment surrounding the cells (Gillespie, 1989).

Pneumolysin (hemolysin) is a soluble protein of 52 kDa produced virtually by all clinical isolates of *S. pneumoniae* (Kanclerski & Mollby, 1987). It is a multifunctional virulence factor encoded by *ply* gene and belongs to the family of cholesterol-dependent cytolysins. The primary sequence of pneumolysin is highly conserved although a

number of variants have been reported (Kirkham *et al.*, 2006; Lock *et al.*, 1996). Upon contact with cell membrane, about 40 pneumolysin monomers oligomerize to form a large transmembrane pore following a series of structural alterations (Tilley *et al.*, 2005).

Pneumolysin plays crucial roles in the disease pathogenesis of *S. pneumoniae* especially in the establishment of pneumonia (Berry *et al.*, 1995; Jounblat *et al.*, 2003; Rubins *et al.*, 1996). This protein enhances the survival of pneumococci in human respiratory tracts and facilitates pneumococcal invasion from lung into the bloodstream leading to bacteraemia (Kadioglu *et al.*, 2002; Orihuela *et al.*, 2004). In the course of bacteraemia, the production of pneumolysin has been linked to higher tendency of overt diseases manifestation (Berry *et al.*, 1999; Orihuela *et al.*, 2004). Conversely, pneumolysin-deficient mutant strains tend to produce chronic sepsis rather than acute infections as shown by Benton *et al*. using an experimental murine model (Benton *et al.*, 1995). On the other hand, the role of pneumolysin in the establishment of pneumococcal meningitis remains debatable (Friedland *et al.*, 1995). However, an increasing body of evidence have shown that not only does pneumolysin play a key determinant role in meningitis (Braun *et al.*, 2002; Wellmer *et al.*, 2002), it is also responsible for various complications that arise from meningitis (Hirst *et al.*, 2004; Hirst *et al.*, 2000).

The native form of pneumolysin is a protective immunogen component. Mice immunized by the purified pneumolysin had significantly higher mean survival time (5.52 days) as compared to the control mice (2.48 days) following intranasal challenge with virulent type 2 pneumococci (Paton *et al.*, 1983). However, native pneumolysin is rather hemolytic and Paton *et al.* had generated two pneumolysin toxoids via site-directed mutagenesis, PdA and PdB. Mice immunized with these toxoids showed enhanced survival time in the immunized mice as compared to the sham-controlled mice (Paton *et al.*, 1991). Alexander and colleagues further demonstrated the potential use of

pneumolysin toxoids as pneumococcal vaccine candidate by showing that PdB conferred non-serotype immune protection in mice (Alexander *et al.*, 1994).

1.2.2. Choline-binding proteins

S. pneumoniae expresses a family of surface-exposed choline-binding proteins (CBPs) which serve as the specific glycoconjugate adhesins against the host cells (Gosink *et al.*, 2000; Rosenow *et al.*, 1997). A representation of pneumococcal cell surface components is shown in Figure 1.1. CBPs are the principal components responsible for pneumococcal cell surface net charge and hydrophobicity to facilitate the adherence of pneumococcus on host cell surface (Swiatlo *et al.*, 2002). The presence of CBPs reduces the electronegative property of the pneumococcal cell. Also, the hydrophobicity of CBPs could substantially be masked by the presence of capsular polysaccharide (CPS) (Swiatlo *et al.*, 2002).

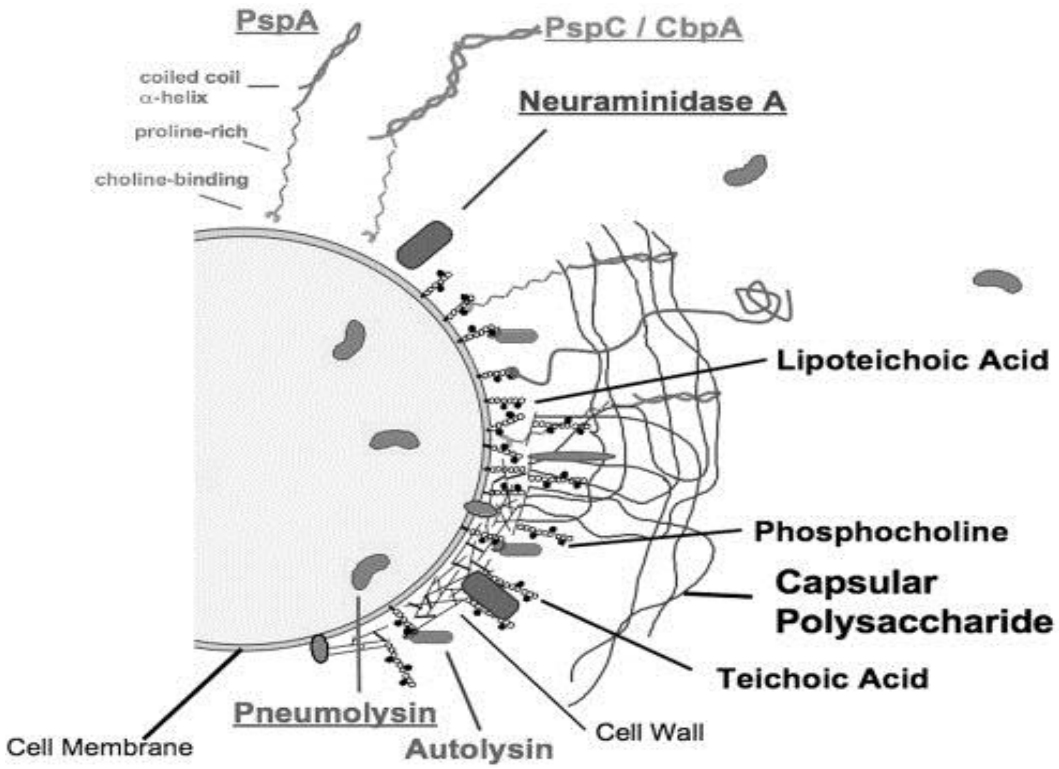


Figure 1.1: A representation of pneumococcal cell surface components (adapted from Bhatty *et al.*, 2011).

Members of CBPs group of proteins include PspA, PspC, and LytA. PspA is

responsible for anti-complementary activity of *S. pneumoniae* by preventing complement fixation of C3 component and complement-mediated opsonization. The inhibitions are suggested to be due to electrostatic repulsion imposed by the highly electronegative termini of PspA protein (Jedrzejas *et al.*, 2001). Also, PspA protects pneumococcus from the bactericidal activity of apolactoferrin protein (iron-depleted form of glycoprotein lactoferrin) by binding to and subsequently inactivates the apolactoferrin (Shaper *et al.*, 2004).

Commonly known as choline-binding protein A (CbpA) and secretory pneumococcal surface protein A (SpsA), PspC is important for adherence and colonization of pneumococci to the epithelial cells of nasopharyngeal (NP) airways and lungs (Balachandran *et al.*, 2002; Rosenow *et al.*, 1997). The protein acts probably by binding to the glycoconjugates of host epithelia such as C3, sialic acid, and lactolactotetraoses (Rosenow *et al.*, 1997; Smith & Hostetter, 2000). Binding of PspC to the polymeric immunoglobulin receptor on the NP epithelial cells which function to transport secretory IgA had been suggested to help in the translocation process of pneumococci across the epithelial and subsequent invasion into other body parts (Zhang *et al.*, 2000a). Moreover, PspC is able to bind specifically to the human secretory immunoglobulin A which reduces bacterial clearance and in turn enhances pneumococcal survival in the host (Hammerschmidt *et al.*, 1997). Similar to PspA, PspC also inhibits C3 component of the complement system (Cheng *et al.*, 2000) and capable of preventing opsonization by binding to factor H which inhibits the formation of C3b of the alternative complement pathway (Quin *et al.*, 2005).

LytA is an autolytic enzyme responsible for cell autolytic activity and the formation and turnover of cell wall. It is functionally homologous to amidase which catalyzes the breakdown of peptidoglycan via N-acetylmuramoyl-L-alanine bond cleavage (Howard & Gooder, 1974). The role of LytA in enhancing the virulence of *S.*

pneumoniae had been demonstrated using the murine bacteraemic and murine pneumonia models (Berry & Paton, 2000; Canvin *et al.*, 1995; Orihuela *et al.*, 2004). This component has recently been targeted to be the potential candidate towards the development of novel therapeutic agent (Rodriguez-Cerrato *et al.*, 2007).

1.2.3. Capsular Polysaccharide

S. pneumoniae is encapsulated by a distinctive layer of CPS which gives the typical smooth and glistening appearance of pneumococcal colonies on solid agar medium known as the "S" cells. The nonencapsulated variants, either due to a lack of capsule expression or as a result of loss of capsule after prolonged growth on artificial medium have a rough appearance termed the "R" cells (Figure 1.2). The oligosaccharides of capsule are synthesized inside the cytoplasm and the polymerized components will be transported and resides on the outermost layer of cell via covalent attachment to the extracellular surface of cell wall C-polysaccharide and peptidoglycan (Sorensen *et al.*, 1990).

CPS produced by the individual strains of pneumococci differs in their chemical compositions which give rise to the classification of serogroups/serotypes based on the antigenic differences immunologically. Serotyping has been conventionally performed using the antisera agglutination method (Quellung reaction). Antigenically-related serotypes (e.g. 9N, 9L, and 9V) are grouped into serogroups while serotypes with no antigenic relatedness are numerically assigned (e.g. 1, 2, 3, and 4). In year 2006, the DNA sequences of capsular biosynthesis genes for all 90 pneumococcal serotypes have been determined (Bentley *et al.*, 2006). The capsular loci are being organized in such a way that genes corresponding to specific serotypes are flanked upstream by *dexB* and downstream by *aliA* conserved genes. These regions are specific to the respective

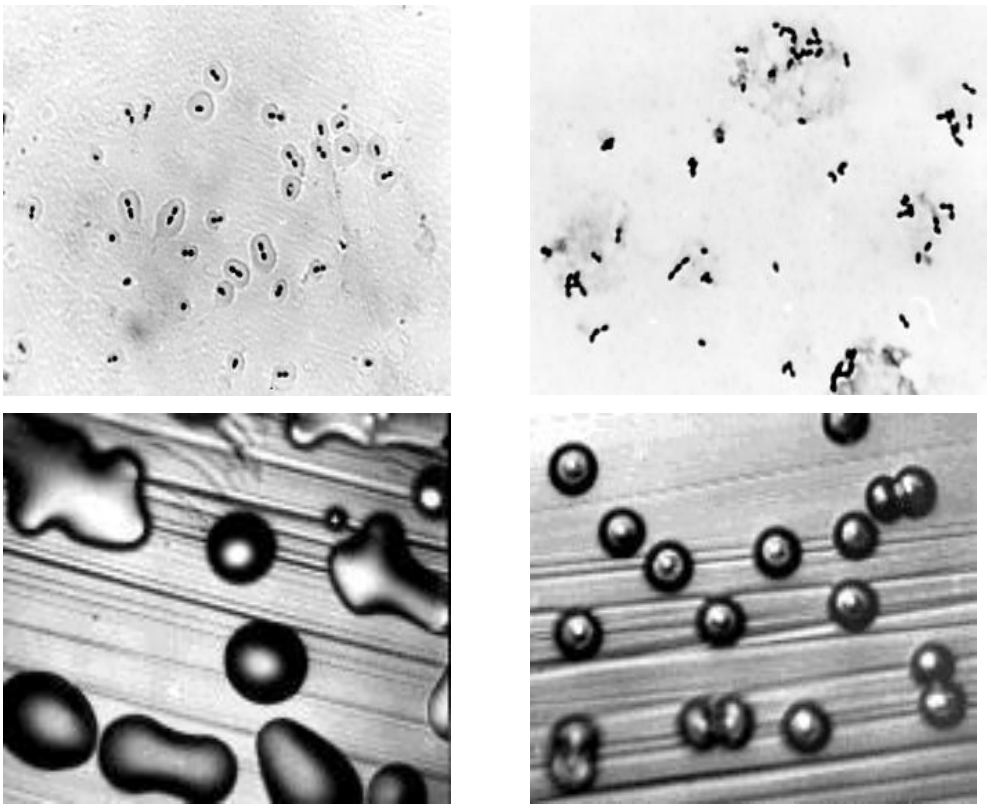


Figure 1.2: Microscopic view of *S. pneumoniae* and the appearance of pneumococcal colonies on solid agar medium. Images on the left shows the encapsulated "S" type cells surrounded by capsule (top) and the smooth and glistening appearance of colonies on agar medium (bottom). Image on the right shows the "R" cells lacking capsule (top) and the rough appearance of colonies (adapted from Austrian, 1953).

serotype/serogroups. Recently, the newer PCR-based multiplex serotyping method has become increasingly common (Njanpop Lafourcade *et al.*, 2010; Pai *et al.*, 2006).

Although phenotypic variations are readily observed for different strains of *S. pneumoniae*, certain serotypes such as the extensively mucoidal serotype 3 colonies appear more distinctive than the other serotypes. CPS represents one of the major virulence determinants in *S. pneumoniae* and can be viewed as a near perfect shield to the bacteria (Austrian, 1981; Henrichsen, 1995). The pneumococcal CPS is usually 200 - 400 nm in thickness and the expression of CPS is indispensable for pneumococcal virulence and serves important roles during the processes of cell adherence and invasion (Briles *et al.*, 1992; Kelly *et al.*, 1994; Sandgren *et al.*, 2005). Highly virulent strains tend to produce thicker capsule as compared to the less virulent strains (Mac & Kraus,

1950). In addition, sterile site isolates are usually encapsulated and much more virulent than the nonencapsulated variants.

Blood-borne pneumococci are extensively cleared by the macrophages which reside in the marginal zone of the spleen (Kang *et al.*, 2004). This process is mediated by the SIGN-R1 receptor which is a C-type lectin present on the macrophage. Absence of SIGN-R1 receptor significantly increases the hypersensitivity of mice against challenge by pneumococci (Kang *et al.*, 2004; Lanoue *et al.*, 2004). Pneumococcus expressing CPS is also strongly antiphagocytic (Cross, 1990). The electrostatic repulsions between the highly charged polysaccharide and the phagocytes interrupts with phagocytes binding (Lee *et al.*, 1991). Also, the capsular layer helps prevent the pneumococci from neutrophil killing by minimizing the neutrophil extracellular trapping effects (Wartha *et al.*, 2007). The presence of CPS also reduces the accessibility of complement components on cell surface and thus prevent complement fixation (Abeyta *et al.*, 2003). It was observed that CPS merely "buried" the complement component iC3b or the Fc region of IgG into deeper surfaces hence preventing the activation of the corresponding receptors on phagocytic cells (Musher, 1992; Winkelstein, 1981). Additionally, CPS enhances the antibiotic tolerability of *S. pneumoniae* by reducing spontaneous or antibiotic-induced cell autolysis (Fernebro *et al.*, 2004). However, the nonencapsulated strains are still able to cause superficial infections such as conjunctivitis (Crum *et al.*, 2004; Martin *et al.*, 2003).

1.2.4. Pneumococcal colonization

Upper airway colonization by *S. pneumoniae* is common and poses no harm to humans. However, colonization represents an important intermediary platform for the horizontal spread of bacteria and marks the initial adherence of pneumococci to the respiratory tract epithelial. It is considered the crucial step preceding further invasion

into deeper tissues leading to severe infections (Di Guilmi & Dessen, 2002; Weiser *et al.*, 1996).

The prevalence of pneumococcal carriage is influenced by several factors including antibiotic usage, day care center attendance, and crowding (Bakir *et al.*, 2001; Bogaert *et al.*, 2004b; Cohen *et al.*, 2006; Rivera-Olivero *et al.*, 2007). NP carriage in children is particularly important as the major reservoir and the source of horizontal disease transmissions among the close contact community members including parents, siblings, and nursery centers (Givon-Lavi *et al.*, 2002; Hussain *et al.*, 2005). NP colonization is more commonly seen in younger children as compared to other age groups. It begins as early as in newborns and one or more strains can be acquired simultaneously or sequentially over time. Subsequently, the rate of colonization increases and reaches a peak of approximately 55% at the age of three followed by a gradual fall thereafter (Bogaert *et al.*, 2004a; Bogaert *et al.*, 2004b). For example, the highest carriage rate had been determined among pre-school children in Malaysia and the prevalence decreased in school-age children (Le *et al.*, 2012). In the developing world, pneumococcal carriage can reach up to 95% in children < 3 years old and approximately 40% among the adults (Austrian, 1986b; Lloyd-Evans *et al.*, 1996). Acquisition of *S. pneumoniae* begins from as young as ≤24 months old (Le *et al.*, 2012).

1.2.5. Pneumococcal infections

For decades, *S. pneumoniae* has been recognized as one of the major human bacterial pathogen responsible for serious invasive pneumococcal infections such as meningitis, sepsis, and pneumonia (Bryce *et al.*, 2005; Gillespie, 1989; Musher, 1992; O'Brien *et al.*, 2009; van der Poll & Opal, 2009). Invasive pneumococcal disease (IPD) is defined as the isolation of *S. pneumoniae* from a normally sterile body site/fluid.

Those at the extreme ages are especially at greater risk of pneumococcal infections (Rohani *et al.*, 2011).

Pneumococcal diseases have been the leading cause of vaccine-preventable deaths (VPDs) among children aged five and below (MMWR Morb Mortal Wkly Rep, 2006) (Figure 1.3). According to World Health Organization (WHO), 0.7 – 1 million deaths out of the 1.6 million total deaths due to pneumococcal diseases were children aged less than five (World Health Organization, 2007). Children from developing countries are the most heavily impacted group by pneumococcal diseases (Scott, 2008). *S. pneumoniae* represents the leading etiological agent causing acute bacterial respiratory infection (ARI) deaths and nonepidemic meningitis in children in developing countries (Mulholland, 1999; Peltola, 2001). In Malaysia, respiratory system infections

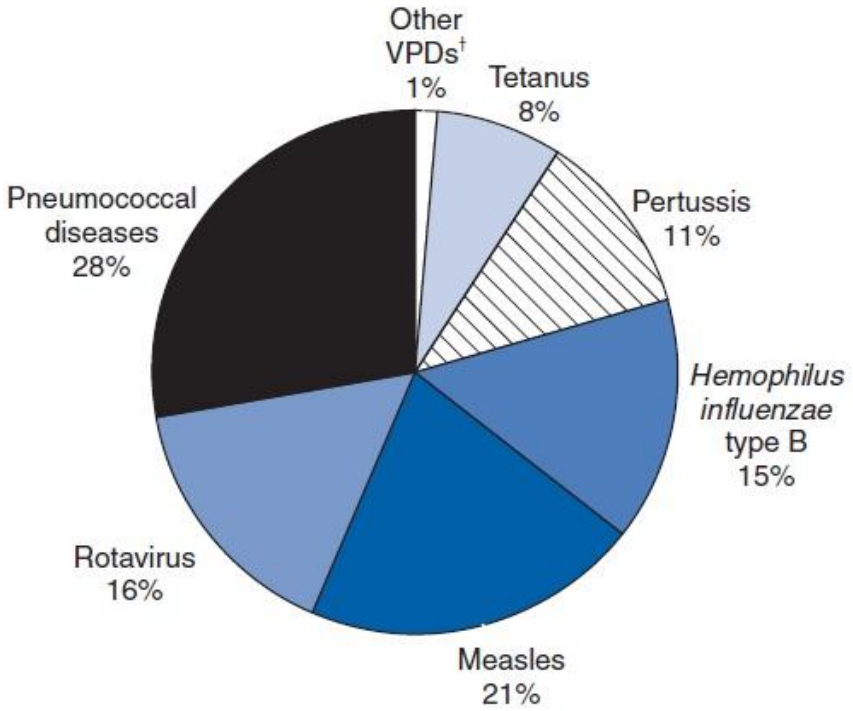


Figure 1.3: Percentage of VPDs according to disease among children < 5 years old in year 2002 (adapted from MMWR Morb Mortal Wkly Rep, 2006).
†Diphtheria, hepatitis B, Japanese encephalitis, meningococcal disease, poliomyelitis, and yellow fever.

particularly pneumonia represent the commonest pneumococcal diseases (Le *et al.*, 2012). Moreover, *S. pneumoniae* is the major etiological agent causing pneumonia and meningitis (Le *et al.*, 2012). Several factors including age of patients (children and elderly adults), severity of illnesses, and underlying disease-causing immnocompromised conditions including human immunodeficiency virus (HIV) infected individuals have the highest mortality rates among the pneumonia and meningitis patients (Klugman *et al.*, 2007; Yu *et al.*, 2003).

Community-acquired pneumonia (CAP) is a common but potentially life threatening infection. Although the majority of the cases are treated as outpatients, younger children and elderly often require hospitalization due to the severity of the condition of patients (Ho *et al.*, 2009). Among all other bacterial pathogens, *S. pneumoniae* represents the leading cause of CAP and the proportion could reached to as high as 50% (Andrews *et al.*, 2003). However, the prevalence of *S. pneumoniae* in CAP appears to vary greatly by countries. In a study involving 127 hospitalized patients presented with CAP in Malaysia, *S. pneumoniae* was still ranked the second most common CAP pathogen after *K. pneumoniae* although it was responsible for only 5.5% of the cases (Liam *et al.*, 2001). Moreover, *S. pneumoniae* had an incidence rate of 75.4/100,000 patients among the Malaysia population irrespective of age of patients and included both invasive and noninvasive isolates (Rohani *et al.*, 1999b).

Pneumococcal pneumonia can occur as a result of pneumococcal invasion into the lower respiratory tract and rapid inflammatory responses ensue immediately leading to acute respiratory tract infection. It had been documented that HIV-1 infection would increase the risk of pneumococcal pneumonia (Valles *et al.*, 2006). In general, two forms of bacterial pneumonia exist: bronchial pneumonia and lobar pneumonia. Bronchial pneumonia is caused by a variety of respiratory pathogens including *S. pneumoniae*. It involves the infection of alveoli to the bronchioles of the bronchial tree.

This usually affects infants, children, and older aged adults. Most often *S. pneumoniae* causes the typical lobar type pneumonia. Other pneumonic patterns such as lobar bronchopneumonia, interstitial, or mixed pneumonia can also be formed (Kantor, 1981; Ort *et al.*, 1983). Lobar pneumonia usually involves a single lobe of the lung but sometime multiple lobes can be simultaneously infected. The infected area will appear as a consolidated mass. Younger adults are more prone to this type of pneumonia.

Pneumococcal pneumonia often leads to secondary bacteremia (bacteraemic pneumococcal pneumonia) which poses greater risks of mortality and morbidity to the patients. In year 2006, *S. pneumoniae* alone caused about 41,400 IPD cases which resulted in 5,000 deaths in the United States, with the majority of them having attributed to bacteremic pneumonia (Centers for Disese Control and Prevention, 2008). Bacteremia can also occur as a result of direct pneumococcal invasion from the initial sites of colonization into the bloodstream. However, this is relatively uncommon. It is estimated that approximately 20 – 25% of pneumococcal pneumonia is associated with bacteremia (Austrian, 1986a; Mufson, 1981), and the mortality rate could reach up to 20% among the infected patients (Balakrishnan *et al.*, 2000; Lim *et al.*, 2001).

Meningitis can be caused by multiple etiological microorganisms. However, bacterial meningitis due to *S. pneumoniae* could result in the higher mortality rate than others. As estimated by WHO's global Burden of Disease project, approximately 100,000 cases of pneumococcal meningitis were reported among children < 5 years of age worldwide and the case fatality rate was more than 50% (O'Brien *et al.*, 2009). In Egypt, *S. pneumoniae* has been a significant pathogen in bacterial meningitis with high mortality rates especially among those < 1 year old (Girgis *et al.*, 1993; Guirguis *et al.*, 1990; Miner & Edman, 1978). Patients recovered from pneumococcal meningitis are also likely to have permanent neurological sequelae (Gouveia *et al.*, 2011).

S. pneumoniae frequently causes some milder manifestations of mucosal infections such as otitis media and sinusitis. Nevertheless, pneumococcal-related arthritis, peritonitis, and pericarditis are relatively rare. Although pneumococcus is not a typical agent in urinary tract infection and report of such cases are extremely rare, sporadic cases of pneumococcosuria have been reported in newborns, children and adult patients (Burckhardt & Zimmermann, 2011; Miller *et al.*, 1989; Nguyen & Penn, 1988).

The pathogenicity and disease-causing ability of *S. pneumoniae* have been suggested to be linked to the genetic background of the pneumococcal strains (Blue & Mitchell, 2003; Kelly *et al.*, 1994; Sandgren *et al.*, 2005). Fortunately, none of the pneumococcal disease state promote pneumococcal transmission, hence suggesting that the virulence of *S. pneumoniae* is directed mainly towards adaptation and establishment of infection which is confined to the primary host rather than being highly contagious (Kadioglu *et al.*, 2008). However, dissemination of the pathogen can occur through direct contact with respiratory secretions from infected person (van der Poll & Opal, 2009).

1.3. Pneumococcal serotype distribution

1.3.1. Pneumococcal vaccinations

Up to date, 93 pneumococcal serotypes have been documented with the latest addition of serotype 11E (Calix & Nahm, 2010) , 6D (Jin *et al.*, 2009) , and 6C (Park *et al.*, 2007). Current preventive strategy against pneumococcal infections relies primarily on the stimulation of serotype-specific immune protection via vaccination. The 23-valent pneumococcal polysaccharide vaccine (PPV23, Pneumovax) is the first pneumococcal vaccine introduced which covers as much as 23 pneumococcal serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F,18C, 19F, 19A, 20, 22F, 23F, and 33F). However, the efficiency of PPV23 vaccination is relatively poor in

children < 2 years of age and in immunosuppressed individual (Overweg *et al.*, 2000). The primary PPV23 target group are the adults (Moberley *et al.*, 2008; Ogilvie *et al.*, 2009; Shapiro *et al.*, 1991; Temple *et al.*, 1991). Although the effectiveness of PPV23 among the elderly is rather debatable, a prospective cohort study involving all 11,241 community-dwelling elderly ≥ 65 years old in Spain studied from January 2002 through April 2005 provided strong arguments for the systematic vaccination of the elderly (Vila-Corcoles *et al.*, 2006). The major outcomes from the study clearly stated that PPV23 essentially prevented pneumococcal pneumonia (with or without bacteremia), reduced the overall rates of pneumonia and the associated mortality although the protective effect against IPD were not statistically significant.

The 7-valent pneumococcal conjugate vaccine (PCV7, Prevnar) comprises of serotype 4, 6B, 9V, 14, 18C, 19F, and 23F. It is formulated by conjugating the serotype antigen to the nontoxic diphtheria proteins CRM_{197} which elicits strong immune responses in infants and younger children < 2 years of age. PCV7 is also recommended for children < 5 years old who are at higher risks of invasive pneumococcal infections including those with immunocompromised conditions like HIV infection, diabetes mellitus, sickle cell diseases, and chronic heart and lung diseases (American Academy of Pediatrics, 2000; MMWR Recommendations and Reports, 2000). In mid 2000, this vaccine was being incorporated as part of the childhood vaccination program in the United States (American Academy of Pediatrics, 2000; MMWR Recommendations and Reports, 2000). This was later followed by several other countries including United Kingdom (Cameron & Pebody, 2006; 2006), Netherlands (Netherlands, 2005), and Norway (Bergsaker & Feiring, 2006). Later, newer formulations with the addition of serotype 1, 5, and 7F over PCV7 vaccine in the 10-valent pneumococcal conjugate vaccine (PCV10, Synflorix, GlaxoSmithKline) and the 13-valent pneumococcal conjugate vaccine (PCV13, Prevnar 13, Pfizer) with further incorporation of serotypes 3,

6A, and 19A over PCV10 vaccine are currently available. Recently, several countries have replaced PCV7 with the newer PCV13 vaccine to provide wider coverage against the six additional vaccine-serotypes (VTs). With the increasing number of VTs covered in the PCVs, further reduction in the reports of IPD cases is anticipated (Flasche *et al.*, 2011). Up to year 2010, PCVs had been introduced in a total of 55 countries (MMWR Morb Mortal Wkly Rep, 2011).

The effectiveness of PCV7 vaccine against the respective VTs varied from 100% (serotype 9V) and 98% (serotype 23F) to 87% (serotype 19F) (Whitney *et al.*, 2006). Apart from that, the efficacies of PCV7 vaccine are influenced by several factors such as doses of vaccine received, age of recipient at the point of vaccination, population coverage of vaccine, and geographical variations of serotype distribution (Hausdorff *et al.*, 2005; Imohl *et al.*, 2010b; Whitney *et al.*, 2006). Interestingly, it was found that the level of antibiotic exposure will affect the efficacy of PCV7 vaccine whereby high PCV7 efficacy was achieved in the US which has low antibiotic exposure environment while the opposite was observed in France which has a relatively high antibiotic exposure environment (Cherry & Woodwell, 2002; Geslin *et al.*, 1998; Guillemot *et al.*, 1998; Karlowsky *et al.*, 2003; Perrocheau *et al.*, 2002; Schuchat *et al.*, 1997; Temime *et al.*, 2005). These studies showed that antibiotic exposure level could have unexpected impact on the efficacy of PCV7 vaccine. On the other hand, it was suggested that the antibiotic selective pressures due to the use of antibiotics could affect the pneumococcal serotype distribution (Moore, 2009; Moore & Whitney, 2008). However, such impact should be minimal (Weinberger *et al.*, 2011).

1.3.2. Pre- and Postvaccination changes in serotype distribution

A number of serotypes including serotype 19F, 23F, and 19A are in general the most prevalent serotypes reported elsewhere (Arredondo-Garcia *et al.*, 2011; Bae & Lee,

2009; Le *et al.*, 2011b; Soh *et al.*, 2000). Following the routine use of PCV7 vaccine, drastic reduction in the incidences of VTs have been widely documented as in the US (Farrell *et al.*, 2007; Grijalva *et al.*, 2007; Kaplan *et al.*, 2004; 2010), Norway (Vestrheim *et al.*, 2008), Germany (Ruckinger *et al.*, 2009), France (Dubos *et al.*, 2007; Lepoutre *et al.*, 2008), Canada (Kellner *et al.*, 2009), and Australia (Roche *et al.*, 2008). The reduced horizontal transmission of VTs from the primary PCV7-vaccinated group would have indirectly benefited the nonvaccinated children and adults in the populations (Lexau *et al.*, 2005; MMWR Morb Mortal Wkly Rep, 2005). This phenomenon is called herd immunity (Hicks *et al.*, 2007; Pilishvili *et al.*, 2010a). Notably, the indirect benefits from PCV7 vaccination have marked the reduction in the prevalence of antibiotic-resistant pneumococci (Dagan & Klugman, 2008; Joloba *et al.*, 2001; Kyaw *et al.*, 2006; Stephens *et al.*, 2005), the multidrug resistant pneumococci (Dias & Canica, 2007), and the incidences of IPD (Kaplan *et al.*, 2004; Whitney *et al.*, 2003) .

The major issue arising following the widespread use of PCV7 vaccine has been the "serotype replacement" whereby the protective coverage conferred by PCV7 against VTs has gradually been diminished by the concomitant rise in the proportions of nonvaccine serotypes (NVTs) (Lipsitch, 1999; Spratt & Greenwood, 2000). Selective pressure from PCV7 vaccine would have eliminated the VTs (Kaplan *et al.*, 2004; Messina *et al.*, 2007), however, the open niches created might attract the expansion of NVTs which possess survival advantage in the microflora to fill up the empty sites (Weinberger *et al.*, 2011). Moreover, due to the competent nature of *S. pneumoniae*, transformation at the intra- or inter-species level can lead to genetic recombination of CPS genes via horizontal genetic material transfer (Coffey *et al.*, 1995b). The recipient cells may then undergo capsular switching leading to subtle change from one serotype to another (Silva *et al.*, 2006). Transformation of VTs to NVTs would allow the

pneumococcal strains to escape the selective pressure by PCVs and enhance the survival fitness of the strains. A large number of studies have found in common the emergence of serotype 19A as well as serotypes 3, 15, 22F, and 33F following the introduction of PCV7 vaccine (Farrell *et al.*, 2007; Mera *et al.*, 2008; Messina *et al.*, 2007; Pelton *et al.*, 2007; Pichichero & Casey, 2007; Richter *et al.*, 2009; Techasaensiri *et al.*, 2010). Most of these studies have also highlighted the increased findings of NVT-associated antibiotic resistance pneumococci. In fact, groups with immunodeficiency or conditions that predispose them to higher risks of pneumococcal infections were found to have higher tendency of pneumococcal diseases due to replaced-serotypes (Pilishvili *et al.*, 2010b). Serotype replacement had also been reported for the carriage serotypes among younger children in the US (Huang *et al.*, 2009). Despite all these, in depth analysis by Weinberger and Whitney strongly suggested that the postvaccination emergence of NVTs did not occur merely by chance and certainly PCV7 vaccination accelerated the rapid increment in NVTs (Weinberger *et al.*, 2011). However, it should be noted that changes in the seroepidemiology can occur as a result of natural background fluctuation (Normark *et al.*, 2001). For instance, temporal fluctuation in serotype 1 had been reported in two studies (Fenoll *et al.*, 2009; Harboe *et al.*, 2010) and increase in serotype 19A had also been reported among the nonvaccinated populations (Choi *et al.*, 2008; Dagan *et al.*, 2009).

Furthermore, PCVs are serotype-specific and the primary strategy in designing newer PCVs focus primarily on increasing the number of serotypes antigen. However, this is also the main shortcoming due to the low number of serotypes being included over the total number of pneumococcal serotypes. There are still unidentified serotypes which indicate that many uncommon and minor serotypes are yet to be discovered.

1.3.3. Serotype-specific pneumococcal virulence

The virulence of *S. pneumoniae* is relatively serotype-specific (Austrian, 1981). A study using mice infected with different serotypes/groups of pneumococci demonstrated the distinctive disease-causing ability of *S. pneumoniae* whereby serotype 4 was found to be highly virulent (100%) followed by serogroup 6 (60%) and serotype 3 (40%) (Briles *et al.*, 1992). In contrast, serogroups 14, 19, and 23 were avirulent. Besides this, the virulence of pneumococci also differs with regards to the routes of challenge. As shown by Briles *et al*., the selected strains of pneumococci were lethal (one serotype 1 and one serotype 6B) when injected via the intraperitoneal (IP) route but failed to infect the mice when given intravenously (IV) (Briles *et al.*, 1992). In humans, serotypes 9 and 14 are frequently associated with IPD while serotypes 6A and 23F are associated with carriage (Zemlickova *et al.*, 2010). In addition, serotype 1 comprises > 6% of IPD cases in various regions of the world (Hausdorff *et al.*, 2000). Notably, serotype 1 has frequently been associated with disease outbreaks (Gupta *et al.*, 2008; Le Hello *et al.*, 2010; Leimkugel *et al.*, 2005; Proulx *et al.*, 2002; Yaro *et al.*, 2006). Serotype 1 disease outbreaks can also occur in overcrowded places and/or alcoholism has been an issue (Dagan *et al.*, 2000; DeMaria *et al.*, 1980; Gratten *et al.*, 1993; Mercat *et al.*, 1991). Apart from that, a Spanish study conducted by Valles and colleagues had highlighted the frequent association of serotype 1 in pneumococcal CAP (Valles *et al.*, 2006). The relative risk of IPD death associated with serotype 1 is low in contrast to serotype 3 which has very high relative risk of death (Martens *et al.*, 2004). Serotype 2 has been highly associated with childhood meningitis among the children in Bangladesh (Saha *et al.*, 2012). On top of serotype 2, Bangladesh has a number of relatively uncommon serotypes among other countries including serotype 1, 12F/A, 5, and 45 which are not part of the PCV13 vaccine formulation. Besides, serotypes including 1, 5, 7F, and 9V have high tendency in causing childhood pneumonia (Greenberg *et al.*, 2011).

1.4. Antibiotic-resistant *Streptococcus pneumoniae*

Antibiotic susceptibility profiling of bacterial pathogens constitutes the routine practice in most diagnostic laboratories of major hospitals. Such information is essential to help the clinical practitioners to decide upon the best choice of empirical treatment for patients and for continuous monitoring of temporal fluctuation in antibiotic resistance of various human bacterial pathogens present in the region. Choices of antibiotics differ by regions and are frequently influenced by the patterns of antibiotic resistance in the respective regions. Escalating incidences of antibiotic-resistant *S. pneumoniae* have been reported throughout the world (Adam, 2002; Jacobs *et al.*, 2008; Reinert, 2009). Of concern, the emergence of antibiotic-resistance especially the multidrug resistant (MDR) *S. pneumoniae* has complicated the disease burden of pneumococcal infections (Lynch & Zhanel, 2009). Treatment failure is associated with increased healthcare financial burden and the risks of morbidity and mortality (Garcia-Vidal & Carratala, 2009; Menendez & Torres, 2007; Menendez *et al.*, 2004; Roson *et al.*, 2004). The course of treatment and patients management have to be modified depending on the antibiotic susceptibility of the strain isolated, current trend in antibiotic susceptibility as reported from case studies and surveillance data, as well as recommendations from the advisory committee of the respective country. Adherence to antibiotic guidelines implemented by the local authorities would be important to prevent discordant therapy (Garcia-Vidal & Carratala, 2009).

Even though many antibiotics are currently available, ß-lactam antibiotics including penicillin, cephalosporins, and carbapenems remain as the preferred choice of antibiotics against pneumococcal infections (Yu *et al.*, 2003). In the case of preliminary failure of ß-lactam antibiotics, other major antibiotic classes including macrolides, vancomycin, and fluoroquinolones can be considered as the alternative options (Mandell *et al.*, 2007). Increasing antibiotic resistance especially with *S. pneumoniae*

has been a major concern as ineffective therapy would delay disease resolution and dramatically increase the risks of complication and death (Mandell *et al.*, 2007).

The treatment outcomes could sometimes be relatively inconsistent with the *in vitro* antibiotic susceptibility profiles of the pneumococcal strains isolated from patient (Feikin *et al.*, 2000; Metlay *et al.*, 2000). A multicenter study conducted by Cardoso *et al*. had observed no significant association between treatment failures and the level of antibiotic resistances of pneumococci isolated from children with severe pneumonia (Cardoso *et al.*, 2008). In addition, the treatment outcomes might also be complicated by other factors such as co-infection by other pathogens or presence of underlying conditions which predispose one to pneumococcal infections. Furthermore, the site of infection and the treatment prescribed do affect the treatment outcome. (Ahronheim *et al.*, 1979; Gartner & Michaels, 1979; Mace *et al.*, 1977).

There are many factors contributing to the widespread antibiotic resistance in *S. pneumoniae*. Generally, the bacteria begins to counteract and develop resistance under environment of strong antibiotic selective pressure especially in hospital and large scale usage of antibiotics in agricultural, veterinary, poultry and animal husbandry feeds for infection preventions and as growth promoters (Davies, 1994). Major outbreaks due to antibiotic resistant *S. pneumoniae* have been documented (Gupta *et al.*, 2008; Leimkugel *et al.*, 2005; Lynch & Zhanel, 2009; Proulx *et al.*, 2002; Yaro *et al.*, 2006). Of utmost concern is the reports of MDR *S. pneumoniae* which was first reported in Johannesburg in 1977 (Jacobs *et al.*, 1978). MDR pneumococci were found to be highly prevalent in Asia (36.3%) as compared to other regions (approximately 5%) (Jones *et al.*, 2003).

1.4.1. β-lactam resistance

Before the availability of effective antimicrobial therapy, the mortality rate in patients with pneumococcal diseases could reach up to 80%. The discovery of ß-lactam antibiotics particularly penicillin had greatly reduced the morbidity and mortality associated with pneumococcal infections. Since then, penicillin has become the drug of choice in empirical therapy of pneumococcal infections (Appelbaum, 1996; Klugman, 1990). Combination therapy with both penicillin and macrolide is sometimes chosen especially when the patient is in critical pneumococcal bacteremic condition (Baddour *et al.*, 2004).

PBPs are produced by many gram-positive and gram-negative bacteria including *S. pneumoniae* as cytoplasmic or membrane-bound proteins. The enzymes catalyze the polymerization and cross-linking of peptidoglycan precursors in bacterial cell wall biosynthesis (Navarre & Schneewind, 1999). *S. pneumoniae* produces a total of six PBPs which are grouped based on their molecular weight: The bifunctional high molecular weight class A PBPs – PBP1A, 1B, and 2A are responsible for transglycosylase and transpeptidase activities; the high molecular weight class B PBPs – PBP2B and 2X function as transpeptidases; and the low molecular weight PBP3 serves as DD-carboxypeptidases. PBPs contain essentially three conserved motifs: Ser-X-X-Lys (SXXK), Ser-X-Asn (SXN), and Lys-Thr/Ser-Gly (KT/SG) motifs (Hakenbeck, 1998).

ß-lactam antibiotics act by acylation of serine residue at the active site of PBPs forming a covalent penicilloyl complex which in turn inactivates the PBPs leading to inhibition of transpeptidase and DD-carboxypeptidase activities (Ghuysen, 1991; Ghuysen & Dive, 1994). Expressions of altered forms of PBPs with reduced affinity against ß-lactam antibiotics can be attributed to mosaic genes that arose from interspecies recombination events (Dowson *et al.*, 1994; Hakenbeck *et al.*, 1980;

Zighelboim & Tomasz, 1980). Such mosaic *pbp* genes possess both regions of high sequences homology to the susceptible strains and regions with diverge nucleotide sequences (Andreu & Rivas, 1998; Coffey *et al.*, 1995b; Dowson *et al.*, 1989; Laible *et al.*, 1991; Martin *et al.*, 1992). It had been suggested that antibiotic pressure can lead to genetic rearrangement in *S. pneumoniae* which causes amino acids alterations and reduced the PBPs affinities toward ß-lactam antibiotics (Sanbongi *et al.*, 2004).

Different PBPs subtypes are associated with resistance against different ß-lactam antibiotics. Pneumococcal resistance against ß-lactam antibiotics is frequently associated with mutations in PBP1A, PBP2B, and PBP2X (Asahi *et al.*, 1999; Barcus *et al.*, 1995; Dowson *et al.*, 1994; Hakenbeck, 1998; Sanbongi *et al.*, 2004). Collectively, these three PBPs confer high level of penicillin-resistance to *S. pneumoniae* (Barcus *et al.*, 1995). In addition, PBP2B is responsible for penicillin and carbapenem resistances whilst cephalosporin resistance is largely encoded by PBP2X (Sanbongi *et al.*, 2004). As shown by Palanisamy *et al.*, all penicillin-susceptible *S. pneumoniae* (PSSP) isolates in the study were found to have unaltered pbp2b gene while the penicillin-intermediate *S. pneumoniae* (PISP) and penicillin-resistant *S. pneumoniae* (PRSP) isolates possessed altered PBP2B gene which contributed to the penicillin resistance (Palanisamy *et al.*, 2008). Notably, PBP1A further enhances the antibiotic resistance in strains with mutations in PBP 2B or PBP2X and mutations of PBP1A and PBP2X are required for high level resistance against the third generation cephalosporins (Coffey *et al.*, 1995a). It was also noticed that PISP and PSSP strains tend to have higher resistance to other β-lactam antibiotics such as cephalosporins while strains susceptible to penicillin were likely to be susceptible to other classes of antibiotic (Palanisamy *et al.*, 2008). Notably, the unique PBP2B restriction fragment length polymorphism (RFLP) pattern observed in pneumococcal isolates with reduced penicillin susceptibility was suggested to be correlated to the resistance level (Desa *et al.*, 2005). In addition, pneumococcal isolates

exhibiting elevated macrolide-resistance similarly displayed unique RFLP patterns (Desa *et al.*, 2005).

Beside PBPs as the principal resistance mechanism, involvement of non-PBP components has also been documented. This includes the putative glycosyltransferase A (CpoA) and histidine protein kinase (CiaH) which confer low level β-lactam resistance (Grebe *et al.*, 1997; Guenzi *et al.*, 1994; Hakenbeck *et al.*, 1999). It was proposed that these non-PBP genes might have significant metabolic role in cell wall components biosynthesis prior to the enzymatic reactions by PBPs (Hakenbeck *et al.*, 1999). In contrast, the murMN genes also influence the susceptibility of pneumococci against β-lactam antibiotics. These two genes display mosaicsm similar to PBPs (Hakenbeck, 1998; Hakenbeck *et al.*, 2001). The MurM enzyme is responsible for the biosynthesis of branched-stem muropeptides in the cell wall of *S. pneumoniae*. Mutation in MurM enzyme is crucial for high level penicillin and cefotaxime resistance in *S. pneumoniae* (Smith & Klugman, 2001). The number of murM alleles has been linked to the degree of muropeptides branching in the cell wall. The extensively branched muropeptides thus altered the cell wall structure and reduced the antibiotic affinity to the pneumococcus (Smith & Klugman, 2001). A recent study had also noticed that the additional murM allele could be the reason for the resistant strain exhibiting lower susceptibility to penicillin (Palanisamy *et al.*, 2009). In addition, the resistant strain was found to have ethanolamine incorporated in the cell wall (Palanisamy *et al.*, 2009). On the other hand, more recently a putative iron permease (spr1178) had also been discovered using the whole genome sequencing approach (Fani *et al.*, 2011).

Rapid emergence of antibiotic resistance has compromised β-lactam antibiotics as the preferred antimicrobial agents against pneumococcal diseases in the future (Jacobs, 2003; Jones, 1999). Penicillin-nonsusceptible *S. pneumoniae* (PNSP) has become increasingly common ever since the first report of penicillin-intermediate *S.*

pneumoniae (PISP) strain in Australia in 1967 (Hansman & Bullen, 1967). The distribution of PNSP has been related to geographical variations (Lynch & Zhanel, 2009). Epidemiological data revealed that rate of PNSP was especially high and could reach up to 50% in Asia (Lynch & Zhanel, 2009) and in two European countries, France and Spain (Adam, 2002; Lynch & Zhanel, 2009; Reinert *et al.*, 2005a). Continued expansion of PNSP strains have been documented (Le *et al.*, 2012). On the contrary, the figures were comparatively low in Germany, Sweden, and Finland (Lynch & Zhanel, 2009). Of major concern is the documentation of PRSP. Indeed, isolates with very-high-level penicillin resistance (> 8 μg/ml) had first been reported in 1995 in the US and the rate has been escalating ever since (Schrag *et al.*, 2004). These strains also displayed resistance against at least three other antibiotic classes and patients of extreme ages (< 5 and > 65 years old) were at higher risks of infection by these strains (Schrag *et al.*, 2004). Following reports of treatment failures in pneumococcal meningitis involving the PRSP strains, the American Academy of Pediatrics and Infectious Diseases Society has recommended the use of third generation cephalosporin to replace penicillin as pharmacokinetic evidences have showed that the concentration of penicillin presence in the central nervous system is inadequate against the PRSP strains (American Academy of Pediatrics Committee on Infectious Diseases, 1997; Begg *et al.*, 1999; Kaplan & Mason, 1998). The high fatality rate associated with PRSP-related meningitis remains a major concern and newer findings have suggested the use of third generation cephalosporin even at low prevalence of penicillin-resistant pneumococci (Gouveia *et al.*, 2011).

In January 2008, the Clinical and Laboratory Standards Institute (CLSI) introduced major revisions in the interpretation of antibiotic susceptibility for *S. pneumoniae*. The penicillin susceptibility breakpoints for nonmeningitis infections (susceptible ≤ 2 μg/ml; intermediate 4 μg/ml; and resistant ≥ 8 μg/ml) had been revised

while breakpoints were unchanged (susceptible ≤ 0.06 μg/ml; intermediate 0.12 - 1 μg/ml; and resistant ≥ 2 μg/ml) for oral penicillin (Clinical and Laboratory Standards Institute, 2008). CLSI also recommends that meningitis and nonmeningitis breakpoints should be reported for pneumococcal isolates obtained other than from the cerebrospinal (CSF) site. As expected, the proportion of nonmeningitis penicillin nonsusceptible isolates reduced considerably. In the US, data gathered from the Active Bacterial Core Surveillance which involved 10 sites for the year 2006 – 2007 revealed that the percentage of IPD nonmeningitis PISP and PRSP reduced from 15.0% and 10.3% to 5.6% and 1.2%, respectively (Centers for Disease & Prevention, 2008). This was accompanied with an increase from 74.7% to 93.2% for PSSP. In meningitis isolates, the reevaluation also categorized the PISP reported previously to be PRSP and thus contributed to the increased percentage of PRSP from 10.7% to 27.5% (Centers for Disease & Prevention, 2008). In addition, according to the previous breakpoints, 33.9% of the pneumococcal isolates reported by Rohani *et al*. were penicillin-resistant and the remaining 66.1% were penicillin-susceptible (Rohani *et al.*, 2011). However, nearly all (99.2%) of the isolates were reclassified as penicillin-susceptible with only 0.84% were penicillin-intermediate according to the new breakpoints. Although the changes would be rather confusing among the clinicians, it is pertinent to note that pneumococcal isolates with reduced susceptibility to penicillin do not always result in treatment failure (Ho *et al.*, 2009).

Nevertheless, conflicting data have been reported with regards to the penicillin susceptibility of *S. pneumoniae* and treatment outcome. A number of oral cephalosporins such as cefaclor, cefixime, cephalothin, and ceftibuten have unsatisfactory effectiveness for infection involving the PRSP strains (Klugman, 2007). A study conducted by Yu *et al*. had determined that 9.6% of the blood cultured pneumococci among the 844 hospitalized patients were penicillin-resistant (minimum

inhibitory concentration, MIC ≥ 2 μg/ml) and 15% were penicillin-intermediate (Yu *et al.*, 2003). However, penicillin resistance did not result in higher mortality rate. Moreover, discordant therapy (i.e. receipt of a single antibiotic with inactive *in vitro* activity against *S .pneumoniae*) with penicillins as well as cefotaxime and ceftriaxone was not associated with mortality. In contrast, cefuroxime-nonsusceptible *S. pneumoniae* caused increased mortality rates. Besides that, concordance of beta-lactam antibiotic treatment was not associated with time required for defervescence and frequency of suppurative complications (Yu *et al.*, 2003).

The association between penicillin susceptibility and serotypes had been frequently reported. In Germany, serotype 19A and 19F are the major serotypes associated with penicillin-resistance (Imohl *et al.*, 2010c). On the other hand, serotypes 23F, 19F, 6B, and 14 are associated with PNSP in Kuwait (Mokaddas *et al.*, 2008) and Oman (Al-Yaqoubi & Elhag, 2011). Furthermore, the resistance pattern of strains from a particular population is more genetically related to the population from the same region. For instance, investigation on the altered *pbp2b* gene among the PNSP isolates from Thailand showed that the isolates were closely resemble those from other Asian countries such as Japan and Korea than the European isolates (Tribuddharat *et al.*, 2010).

1.4.2. Macrolide resistance

Macrolide antibiotics are characterized by the presence of a large macrocyclic lactone ring. Also known as the macrolide rings, they are either 14-, 15-, or 16-membered structure. This group of antibiotic includes erythromycin A, azithromycin, clarithromycin, and ketolides. Macrolides inhibit protein biosynthesis in bacteria via irreversible binding to the 50S ribosomal subunit which interrupts the peptidyltransferase activity leading to inhibition of peptidyl tRNA transpeptidation and translocation.

Pneumococcal macrolide resistance involves the target modification by ErmB pathway and the macrolide efflux pump Mef/Mel pathway (Ambrose *et al.*, 2005; Leclercq & Courvalin, 2002; Sutcliffe *et al.*, 1996). The ribosomal methylase ErmB encoded by *erm*(B) gene catalyzes the specific adenine dimethylation of 23S rRNA at position 2058 (A2058) (Leclercq & Courvalin, 2002; Weisblum, 1995). This confers high level macrolide-lincosamide-streptogramin B resistance to *S. pneumoniae* harboring *ermB* gene (MLSb phenotype) (Lambert, 2005). It was reported that macrolide-resistance was especially high among the European countries and high proportion (more than 80%) of erythrocymin-resistant strains was due to *erm*B-mediated resistance (Reinert *et al.*, 2005b).

The second mechanism Mef/Mel membrane-bound efflux pump activity is rather specific for 14- and 15-membered macrolides such as erythromycin and its derivatives (e.g. azithromycin) but not clindamycin or streptogramins (Daly *et al.*, 2004; Tait-Kamradt *et al.*, 1997). The aggregation of 12 transmembrane protein domains on the cell membrane produces active efflux machinery which is driven by proton motive force (Clancy *et al.*, 1996). There are two Mef variants, *mefA* or *mefE* (Tait-Kamradt *et al.*, 1997). The prevalence of these variants differ by geographical distribution whereby *mefE* was more prevalent in Asia, US, and North and South America but *mefA* was the predominant efflux mechanism in Canada and parts of Europe (Amezaga *et al.*, 2002; Daly *et al.*, 2004; Hoban *et al.*, 2001; Song *et al.*, 2004a). Low level pneumococcal resistance against the 14- and 15-membered macrolides is due to MefA active efflux mechanism (Weisblum, 1995). High level erythromycin resistance is associated with the alteration of drug-binding sites encoded by *erm*B gene (Descheemaeker *et al.*, 2000; Weisblum, 1995). In Malaysia, *S. pneumoniae* nonsusceptible to the macrolide antibiotic erythromycin were attributed to either *ermB* (59.7%) or *mefE* (35.5%) (Palanisamy *et al.*, 2008). A previous study had also reported that pneumococcal

isolates with higher level of erythromycin resistance (MIC > 8 µg/ml) tend to possess *ermB* in standalone form or exist together with *mefE* (Desa *et al.*, 2005).

An increasing body of evidences have reported the potential risks of clinical failures even with pneumococci of low level macrolide-resistant (Daneman *et al.*, 2006; File, 2006; Iannini *et al.*, 2007; Lonks, 2004; Lonks *et al.*, 2002) (Klugman, 2007). A prospective, population-based surveillance conducted between 2000 and 2004 involving 1696 episodes of pneumococcal bacteremia in Canada reported that isolates with higher macrolide tolerability were associated with higher risk of macrolide failure but the risk of treatment failures did not increase further with erythromycin MIC of > 1 µg/ml (Daneman *et al.*, 2006). Moreover, the study group also noticed that macrolide-resistant pneumococci were significantly more common in pneumococcal bacteremia cases after the failure of macrolide treatment as compared to those without prior antibiotic therapy or treatment using non-macrolide agents (Daneman *et al.*, 2006). Similarly, a large series macrolide therapy failure had been documented from a retrospective, multicenter study involving 122 inpatient CAP patients due to *S. pneumoniae* whereby they observed increased macrolide resistance (> 60%) with inpatient mortality of 5.7% (Iannini *et al.*, 2007). As high as six out of the seven patients who died were infected with macrolide-nonsusceptible strains (Iannini *et al.*, 2007). Among 3,885 patients with chronic obstructive pulmonary disease, it was found that the likelihood of disease progression to CAP was significantly associated with *S. pneumoniae* and ineffective antibiotic therapy (File *et al.*, 2009). PNSP also displays higher tendency to become resistant against other non-ß-lactam antibiotics as well (Lynch & Zhanel, 2009). In the US, it was reported that a high proportion of strains with penicillin resistance (34.2%) were determined to have accompanying erythromycin (31.9%) and trimethoprim-sulfamethoxazole (29.5%) resistances (Doern *et al.*, 2005).

The serotype-specific association of macrolide-resistance with invasive pneumococcal strains has also been documented. In Germany, serotype 14 was found to be highly macrolide-resistant (Imohl *et al.*, 2010a). From the same study as well, children were most frequently affected by macrolide-resistant strains as compared to adults (Imohl *et al.*, 2010a). In addition, 60% of the invasive serotype 14 isolates reported by Clarke *et al*. were erythromycin-resistant (MIC range 1 – 24 μg/ml) and of high clonal relatedness whereby 95% of these erythromycin-resistant serotype 14 isolates belong to ST9 (PMEN England^{14}-9 clone) (Clarke *et al.*, 2004). Nevertheless, it was serotype 19F that was the most common macrolide-resistant strains in Lebanon (Taha *et al.*, 2012). A recent study in Arizona had reported as high as 52% of the macrolide-resistant pneumococci were dual *mefE*/*ermB*-positive from the highly disseminated CC271 multidrug resistant clone carrying the transposon Tn*2010* (Bowers *et al.*, 2012). CC271 is a major multidrug lineage worldwide and constituted a significant portion of macrolide-resistant pneumococci reported in many countries especially Europe and Asia (Li *et al.*, 2011; Reinert *et al.*, 2008; Siira *et al.*, 2009; Xu *et al.*, 2010).

1.4.3. Fluoroquinolones resistance

Fluoroquinolones are derived from and have an additional fluorine atom attached to the central ring structure of the broad spectrum antibiotic quinolones. This class of antibiotics inhibits DNA replication by targeting the DNA gyrase and topoisomerase IV, both of type II DNA topoisomerases. DNA gyrase has two A and two B subunits which are encoded by *gyrA* and *gyrB* genes, respectively and is responsible for ATP-dependent negative supercoiling of DNA (Wang, 1985). Topoisomerase IV serves important roles in chromosomes partitioning during cell division (Luttinger, 1995). The ParC and ParE subunits are encoded by the closely-linked *parC* and *parE* genes, respectively.

Fluoroquinolones exhibit enhanced activity against gram-positive bacteria. Fluoroquinolones resistance in *S. pneumoniae* can be acquired through point mutation, intraspecies recombination, or interspecies recombination with *S. mitis* (Balsalobre *et al.*, 2003; Bast *et al.*, 2001; Fenoll *et al.*, 2000; Stanhope *et al.*, 2005). The mechanism occurs in a sequential, stepwise manner and attributed mainly to specific mutations with *gyrA* or *parC* gene in the quinolone resistance-determining regions (QRDRs) (Gootz *et al.*, 1996; Janoir *et al.*, 1996; Munoz & De La Campa, 1996; Tankovic *et al.*, 1996). First mutation in either *parC* or *gyrA* gene usually causes only low level resistance, however, subsequent second mutation in the other gene eventually lead to high level resistance (de la Campa *et al.*, 2009; Janoir *et al.*, 1996; Patel *et al.*, 2010). For example, Pan *et al*. showed that *S. pneumoniae* with low level ciprofloxacin resistance had *parC* mutation but not *gyrA* whilst strain with high ciprofloxacin resistance had both *parC* and *gyrA* mutations (Pan *et al.*, 1996). This preferred first targeting of either gene is termed primacy (Fisher & Heaton, 2003). Other fluoroquinolones with similar mechanisms of resistance in *S. pneumoniae* include trovafloxacin, norfloxacin, and levofloxacin (Fukuda & Hiramatsu, 1999). On the contrary, newer fluoroquinolones including gatifloxacin and sparfloxacin showed primacy against DNA gyrase (Fukuda & Hiramatsu, 1999; Pan & Fisher, 1997).

Apart from that, the involvement of a putative reserpine-sensitive efflux mechanism encoded by the *pmrA* gene has been described which confers low-level fluoroquinolone resistance in *S. pneumoniae* as compared to topo IV and gyrase mutations (Baranova & Neyfakh, 1997; Brenwald *et al.*, 1997, 1998; Zeller *et al.*, 1997). However, the expression of *pmrA* alone could not explain the reduced ciprofloxacin susceptibility (Palanisamy *et al.*, 2007; Piddock *et al.*, 2002). The pmrA efflux pump was suggested to be rather specific to the older fluoroquinolones such as norfloxacin but not the newer fluoroquinolones such as ciprofloxacin, gatifloxacin, levofloxacin, and

others. Other efflux mechanisms could have involved simultaneously to produce the resistant phenotype (Palanisamy *et al.*, 2007).

Following the escalating incidences of MDR *S. pneumoniae* particularly against the empirical antibiotics β-lactam and macrolides, fluoroquinolones such as levofloxacin, moxifloxacin, and gatifloxacin represent the alternative agents of choice in treatment of pneumococcal infections (Bartlett *et al.*, 1998; Mandell *et al.*, 2003). Unfortunately, strong antibiotic selective pressures after years of widespread fluoroquinolone usage have eventually lead to expansion of fluoroquinolone-resistant *S. pneumoniae* (Chen *et al.*, 1999). In the US, antimicrobial susceptibility surveillances through year 1994 – 2003 conducted by Doern *et al*. had determined that while resistances to major antibiotic classes including ß-lactams, tetracyclines, macrolides, and other drugs were less common, fluoroquinolones resistance have expanded dramatically over the years (Doern *et al.*, 1999; Doern *et al.*, 1996; Doern *et al.*, 2001; Doern *et al.*, 2005). High fluoroquinolone resistance rates have been reported in Spain (7%) and several Asian countries/regions which include Hong Kong (14.3%), Sri Lanka (9.5%), Philippines (9.1%), and Korea (6.5%) (Canton *et al.*, 2003; Jones *et al.*, 2003; Mendes *et al.*, 2004; Perez-Trallero *et al.*, 2001; Song *et al.*, 2004b). Although the pneumococcal isolates are being determined as fluoroquinolones-susceptible, however, the isolates would have acquired single-step mutation and become resistant dramatically upon acquired further mutation as had been reported by Lim *et al*. whereby 59% of the levofloxacin-susceptible pneumococci indeed possessed single-step mutation in the QRDR (Lim *et al.*, 2003).

Reports of fluoroquinolone-resistant pneumococci associated treatment failure have been increasingly documented though uncommon (Ambrose *et al.*, 2004; de la Campa *et al.*, 2003; Kays *et al.*, 2002). The elderly adult group was found to be prone to infection by fluoroquinolone-resistant pneumococci than the children (Yokota *et al.*,

2002). In addition, it has been suggested that treatment failures are more likely to occur among the elderly patients with pneumococcal respiratory tract infections presented with co-morbid diseases and a recent episode of fluoroquinolones (Fuller & Low, 2005). This is likely to occur in strains with first-step mutation in the *parC* or *gyrA* genes (Fuller & Low, 2005). Older generations of fluoroquinolones including ofloxacin and ciprofloxacin should no longer be used in respiratory tract infection whether or not is due to *S. pneumoniae* (Ho *et al.*, 2004; Ho *et al.*, 2001). Moreover, initial treatment with fluoroquinolones in CAP is likely to have lower risk of treatment failure as compared to other antibiotics recommended by the guidelines (Menendez *et al.*, 2004; Roson *et al.*, 2004). Comparing the rates of treatment failure among 3,994 patients based on claims analysis in the US it was noticed that treatment failures associated with levofloxacin was significantly low (Hess *et al.*, 2010). The finding was supported by a previous study which was also claims-based, reporting that patients treated with levofloxacin experienced lower probability of treatment failure (Ye *et al.*, 2008).

1.5. Antimicrobial peptides

1.5.1. General properties of antimicrobial peptides

Living in a complex environment, the organisms are constantly exposed to and interact with other life forms. However, this includes microbial pathogens that can cause considerable harm to the host. As the first line of defense, the host innate immunity must be able to recognize and clear microbial invasions efficiently well before the acquired immune system is being stimulated. In many circumstances, it is important to have the pathogens eliminated without the need to stimulate inflammatory mechanism or the activation of acquired immunity in order to prevent overwhelming immune responses in the host (Bals, 2000). To achieve these, one of the crucial components

expressed by mammalian innate immunity is the production of antimicrobial peptides (AMPs).

AMPs are generally short in length (12 - 50 amino acids), low molecular weight, amphipathic, contain multiple hydrophobic residues, and positive in charge (Brogden, 2005; Hancock, 1997b; Hancock & Chapple, 1999; Nicolas & Mor, 1995; Oren & Shai, 1998). A number of anionic AMPs have also been reported despite being relatively less common than cationic AMPs (Brogden *et al.*, 1997; Harris *et al.*, 2009). AMPs are produced by a vast variety of living organisms ranging from mammals, fish, amphibians, reptiles, arachnids, plants, fungi, and bacteria (Hancock & Scott, 2000; Stark *et al.*, 2002). In humans, AMPs belong to three major classes: cathelicidin, defensins, and histatin. Large number of AMPs have been isolated and characterized in the past decades (Hancock & Chapple, 1999). To date, more than 1,000 AMPs have been identified and a lot more are to be expected in the future. Although multiple isoforms of AMPs with wide sequential and structural variances have been reported (Bulet *et al.*, 2004; Zhu & Gao, 2009), the biological activity of AMPs are generally characterized by the universal structural signatures and sets of molecular biophysical traits (Yount & Yeaman, 2004).

AMPs exhibit broad spectrum antimicrobial activity against wide diversity of gram-positive and gram-negative bacterial species, fungi, as well as eukaryotic parasites and enveloped viruses (Epand & Vogel, 1999; Hancock, 2001; Kamysz *et al.*, 2003; Nizet, 2006). AMPs are selectively crafted into effector compounds with specific and high order functionality in the host innate defense system. They are able to function without memory and high specificity. It has been postulated that the immune system of mammals and other higher organisms has been changing rapidly throughout the evolution to adapt to the ever-changing microbial ecology surrounding and within the host (Lynn *et al.*, 2004).

AMPs can be supplemented by immune cells (mast cells, dendritic cells, macrophages, neutrophils) recruited to the injury sites during acute inflammatory response (Di Nardo *et al.*, 2003; Rosenberger *et al.*, 2004; Turner *et al.*, 1998). Presence of local stimuli such as lipopolysaccharides (LPS) and lipoteichoic acids (LTAs) during bacterial infections may also activate the production of AMPs (Di Nardo *et al.*, 2003; Diamond *et al.*, 1996; Russell *et al.*, 1996). These compounds serve multiple roles in the immune system as immunomodulating agents during the cell's inflammatory response, chemotaxis, cytokine release, cell proliferation, as well as tissue repair (Bateman *et al.*, 1991; Boman, 1995; Elsbach, 2003; Koczulla & Bals, 2003; Koczulla *et al.*, 2003). One of the examples is human cathelicidin LL-37 which functions as human monocytes, T cells and mast cells chemoattractant (Niyonsaba *et al.*, 2002) (De *et al.*, 2000), regulation of chemokine production (Scott *et al.*, 2002), and acts as a potent antiendotoxic agent (Scott *et al.*, 2000). Eventually, the coordinated interactions between AMPs and various immune components lead to clearance of pathogens and enhanced tissue repair processes. Further invasion into deeper sites is prevented, thus inhibits the development of invasive diseases. The production of AMPs has been demonstrated to confer resistance against bacterial infections and enhanced the survival fitness of host (Cheung *et al.*, 2008). Deficiency in AMPs are increasingly being recognized as underlying factors contributed to manifestation of diseases and predispose one against infections (Zaiou, 2007). Recently, the role of AMPs in wound healing (Heilborn *et al.*, 2003) and anticancer activity (Hoskin & Ramamoorthy, 2008; Lee *et al.*, 2008; Mader *et al.*, 2005) have been described. The potential benefits of AMPs as disease enhancement agent have also been recognized in the agricultural and aquacultural industry (Jia *et al.*, 2000). Of note, the transgenic expression of plants with AMPs-encoded genes have shown great agricultural application potential whereby the transformed plants were found to exhibit enhanced resistance against a variety of

phytopathogens and better productivity (Lee *et al.*, 2011a; Oard & Enright, 2006; Rajasekaran *et al.*, 2012).

Secretory AMPs together with other molecules of innate immunity constitute the major components of host mucosal immunity. These AMPs are being released into the oral cavity, gastrointestinal tract, and respiratory tract to protect the mucosal surfaces from potentially harmful microbes and to maintain a steady state common microflora (Lee *et al.*, 2004a; Zasloff, 2002c). In humans, secretory AMPs are mainly cathelicidin and α- and β-defensins (Agerberth *et al.*, 1999). They are produced ribosomally and subjected to further optional posttranslational modifications that are fundamental for optimal activity and exportation. Secretory AMPs are produced by mucosal epithelial cells and skin keratinocytes at the baseline level which are tightly regulated by a sophisticated regulation system (Braff *et al.*, 2005a; Braff *et al.*, 2005b; Dorschner *et al.*, 2001; Nizet *et al.*, 2001). The expression of secretory AMPs are being induced dramatically during the inflammatory response in the events of breaching of skin layer or in response to infection stimuli (Schonwetter *et al.*, 1995).

1.5.2. Structural classes

AMPs consist of a diverse family of polypeptides which can be subgrouped into four main structural classes (Agerberth *et al.*, 1991; Harwig *et al.*, 1995a; Harwig *et al.*, 1995b): (a) amphiphilic α-helical peptides lacking cysteine residue. For examples, mammalian cathelicidins, insect-derived cecropins, and magainins from the skin of *Xenopus laevis* (b) Linear peptides predominated with one or more amino acid types such as mammalian PR-39, prophenins from porcine leukocytes, and Bac 5 and Bac 7 from bovine neutrophils (c) Amphiphilic disulfide-bonded ß-sheet peptides (cysteine-rich) such as porcine protegrins, tachyplesins from horseshoe crab, and mammalian

defensins (d) loop-structured peptides with cysteine-disulfide ring including bactenecin, brevinins, and ranalexin.

1.6. Mechanism of actions

1.6.1. Membrane-active peptides

Bacterial cell wall and outer capsule represent the significant barriers against any incoming antibiotic or foreign substance that might cause considerable damage to the cell. Hence, it is generally considered that AMPs with broad spectrum antimicrobial activity must be able to traverse the protective layers (e.g. CPS in *S. pneumoniae*) whether to target the membrane directly or entering the cytosol to allow subsequent intracellular activity. For intracellular targeting AMPs, it appears that the AMPs would exert certain disruption effects during the transition process although there might not be detrimental to the cells. This also describes the functional duality of AMPs.

The most widely accepted model of AMPs mode of killing is the non-receptor mediated membrane lytic mechanism (Bessalle *et al.*, 1990; Wade *et al.*, 1990). Most of the studies investigating AMPs mode of actions showed that AMPs exert its microbicidal activity by permeabilizing and depolarizing the membrane as the principal killing strategy (Boniotto *et al.*, 2003; Lehrer *et al.*, 1989; Shai, 2002; Yeaman & Yount, 2003). In fact, AMPs binding and interaction with the cell membrane had been linked to bactericidal potency (Lehrer *et al.*, 1988; Lehrer *et al.*, 1985; Tran *et al.*, 2002).

The first contact between AMPs and the target bacteria is essentially driven by electrostatic interactions (Brogden, 2005; Hancock & Chapple, 1999; Nicolas & Mor, 1995; Vaara, 1992). The electrostatic interaction is due to the opposite charge attraction between the cationic AMPs and the negatively charged bacterial cell surface due to the presence of teichoic acids (TAs) and LTAs in gram-positive bacterial cell wall and LPS in gram-negative bacterial cell wall. This occurs irrespective of whether the AMPs are

targeting cell membrane only for killing activity or will eventually be translocated across the membrane to inhibit intracellular components or both. The AMPs initial binding to cell membrane will cause a slight disruption in both the packing of outer membrane and phospholipids chain arrangements in a way termed "self-promoted uptake" (Hancock, 1999). Moreover, when the peptide-bound outer leaflet of membrane flips inward, the peptides draw along the adjacent lipid components create a slight membrane disruption that affects the integrity of the membrane (Matsuzaki *et al.*, 1998). On the other hand, AMPs that exert antimicrobial activity by the formation of pores or ion channel (pseudoionophore) on cell membranes are able to immediately insert themselves into the lipid bilayers. Subsequently, the AMPs interact with the membrane by pore formation or mechanisms that perturb the membrane integrity (Hancock, 1997a; Hancock & Rozek, 2002; Koczulla & Bals, 2003; Yeaman & Yount, 2003; Zasloff, 1992). This will eventually lead to transmembrane channel formation and membrane lysis which in turn causes irreversible osmotic-colloidal interactions leading to drastic and detrimental lost of proton motive forces (Huang, 2000; Oren & Shai, 1998). Besides this, some AMPs are able to form voltage-dependent channel. As in the case of HNP-3, the channel form on planar lipid bilayers is voltage-dependent and weakly anion selective that is linked to the antimicrobial activity observed with HNP-3 (Kagan *et al.*, 1990).

On top of this, an increasing body of evidence had underlined the importance of hydrophobicity in modulating the interaction between AMPs and microbial surface (Hancock & Chapple, 1999; Vaara, 1992). Aggregation of amphipathic AMPs on membrane surface is particularly important for its activity (Hwang & Vogel, 1998; Oren & Shai, 1998). These peptides are able to adopt an amphiphatic structure which segregates the hydrophilic and hydrophobic residues to opposite sides upon coming in contact with the biological membrane (Nicolas & Mor, 1995; Shai, 1999; Tossi *et al.*,

2000). Amphiphilic and lateral amphiphilic (hydrophobic and hydrophilic face toward opposite sides) are required to form hydrophilic ion channel while remain in hold with the adjacent hydrophobic components of cell membrane particularly the lipid moieties. This transmembrane channel is essentially α-helical to facilitate penetration into the membrane. Notably, the α- and ß-defensins characterized up to date show an amphiphilic topology which is suggested to be critical for their bactericidal activity. Lateral amphipathic peptide should have periodic interval of hydrophobic residues per circumference of a helical turn which is approximate to be 3.6 residues.

The cell selectivity of AMPs is suggested to rely on the difference in membrane lipid composition favoring the invading microbes over host cells (Oren & Shai, 1998) (Teixeira *et al.*, 2012). Eukaryotic cells are predominated by zwitterions and sphingomyelin phospholipids which give lower electrostatic affinity towards the cationic AMPs. Therefore, the host cells manage to escape unwanted binding that would cause cell toxicity otherwise. Several AMPs including the magainins, cecropins, and seminal plasmin display high bacterial cell selectivity over mammalian cells (Sitaram & Nagaraj, 1993; Wade *et al.*, 1992; Wieprecht *et al.*, 1997). Insufficient cell selectivity by LL-37, indolicidin, melittin, tachyplesins, protegrins, and others rendering the AMPs toxic to host cells (Blondelle *et al.*, 1993; Kokryakov *et al.*, 1993; Matsuzaki *et al.*, 1997b; Oren *et al.*, 1999; Subbalakshmi *et al.*, 2000). Thus, in order to achieve high and rapid microbicidal effects while avoiding the problem of cell toxicity, immune cells expressing cathelicidins and defensins will release very high local dose of AMPs to initiate immediate microbial killing at the injury sites followed by immediate breakdown of the unwanted remains of AMPs mainly via proteolysis after utilizing AMPs against the microbes (Hancock *et al.*, 2012; Teixeira *et al.*, 2012; Zaiou, 2007).

There are three widely accepted models of membrane-active AMPs: toroidal-pore model, barrel-stave model, and carpet model.

Toroidal pore model

In the toroidal pore model (Figure 1.4), first binding of AMPs to the membrane is followed by cascade aggregation of more peptide units. This action disintegrates the horizontal lipid components of the same membrane and subsequently causes the lipid moieties of outer and inner membrane to fold inwards forming a continuous channel (Brogden, 2005; Hale & Hancock, 2007). Each pore is lined by a number of peptide monomers that are tightly associated with the lipid headgroups making up the wall of the pores throughout the whole pore-forming process (Hale & Hancock, 2007). As a result, cell death occurs due to detrimental leakage of cytoplasmic contents to the surrounding. Example of toroidal pore forming AMPs are magainin 2 (Yang *et al.*, 1998) and melittin (Sengupta *et al.*, 2008). Recently, the first discovery of a gram-positive bacteria-producing toroidal pore forming bacteriocin, lacticin Q had been reported (Yoneyama *et al.*, 2009).

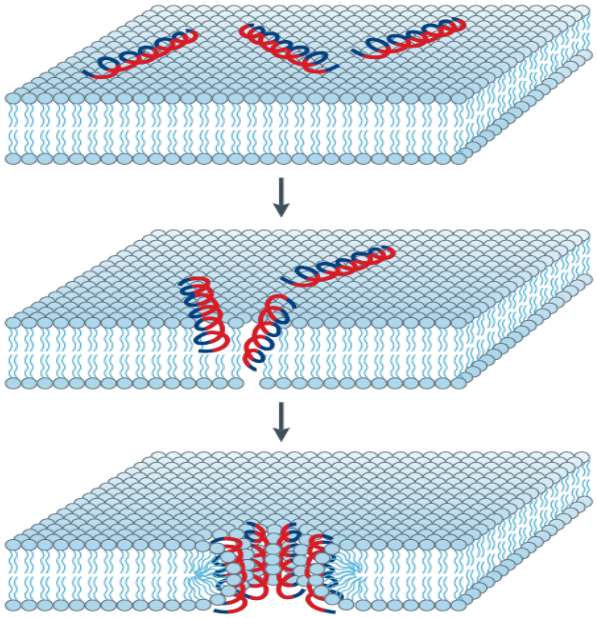


Figure 1.4: The toroidal pore model of AMPs (adapted from Brogden, 2005).

Barrel-stave model

The barrel-stave model is quite similar to toroidal pore model in that both form a transmembrane pore each lined by multiple peptide monomers at the end of the process (Figure 1.5). However, several intermediary steps differ (Brogden, 2005; Hale & Hancock, 2007). The peptide monomers first bind to the membrane and insert themselves in a parallel formation to the lipid components of the membrane. Insertion can occur either by initial single monomer insertion into the membrane followed by lateral coalescence or through lump sum direct insertion of the aggregated oligomer. Hydrophilic side of the peptide monomer will point inward forming the lumen of the channel whereas the hydrophobic side will face outward from the channel in tight association with the lipid core of the bilayers. The stable pore then conduct the leakage of intracellular contents leading to cell death. Alamethicin is one of the many barrel-stave forming AMPs (Yang *et al.*, 2001).

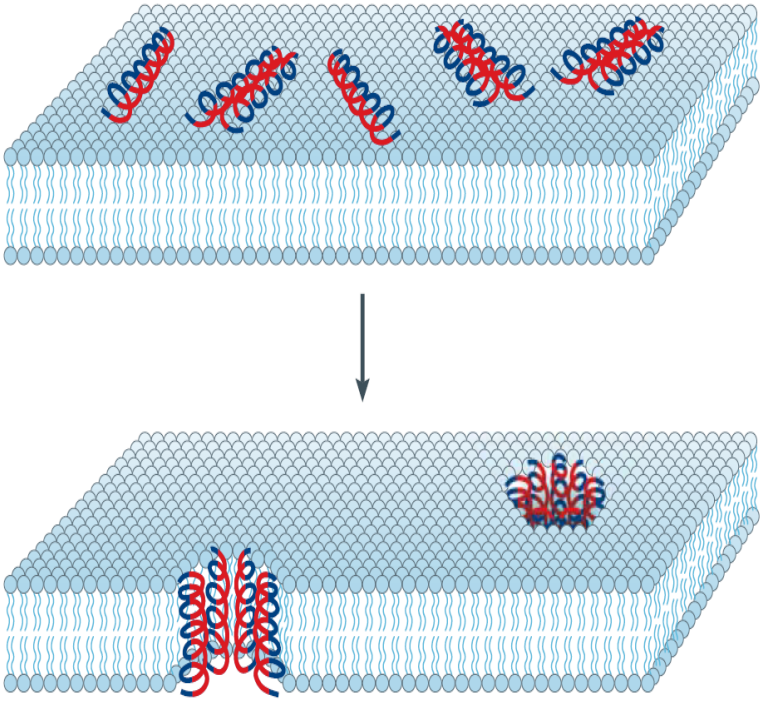


Figure 1.5: The barrel-stave model of AMPs (adapted from Brogden, 2005).

Carpet model

Unlike the two pore forming mechanisms abovementioned, carpet model has no actual transmembrane pore or direct insertion of AMPs into the membrane (Figure 1.6). Instead, the AMPs remain associated with the outer leaflet of cell membrane. When the accumulated peptide monomers on the membrane surface are sufficient to disrupt the membrane electrostatic integrity, the peptides then induce local weakness in the membrane and eventually disintegrate the membrane in a micellar configuration known as the "carpet-like" formation. The leakage of cellular contents thus leads to cell death (Brogden, 2005; Hale & Hancock, 2007). Example of AMPs include the cecropin P1 from porcine intestines (Gazit *et al.*, 1995).

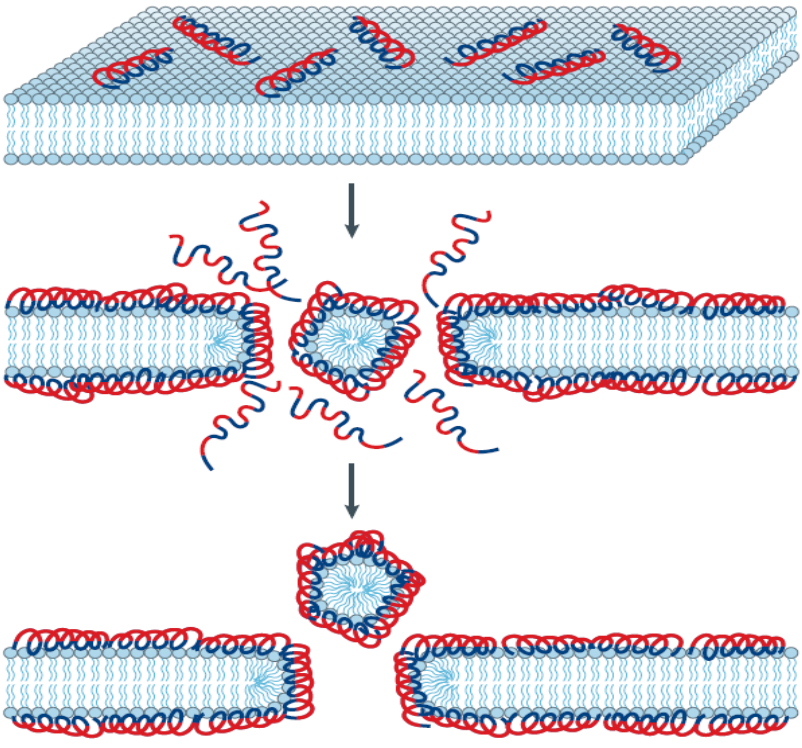


Figure 1.6: The carpet model of AMPs (adapted from Brogden, 2005).

1.6.2. Non-membrane targeting mode of actions

Apart from the membrane active models, there have been increasing reports of intracellular targeting AMPs in the past decades (Brogden, 2005; Hale & Hancock, 2007; Nguyen *et al.*, 2011). Rather than recognizing specific single target site, AMPs are able to trigger multiple inhibitory and lethal events in the same cell whether by membrane or non-membrane targeting mechanism in a cooperative manner to ensure efficient and immediate killing (Nguyen *et al.*, 2011; Peschel, 2002; Powers & Hancock, 2003). A peptide that exerts bactericidal effect via membrane disruption might in other hand kill other species of bacteria using non-membrane targeting actions (Hale & Hancock, 2007). The mechanism of actions might as well be differed vastly between gram-positive and gram-negative bacteria due to the bacterial cell wall structural and compositional differences. Apart from these, AMPs with widely dissimilar primary sequence and solution structure can share similar bactericidal pathway as seen with histatin 5 and HNP-1 (Edgerton *et al.*, 2000). A number of major non-membrane targeting mechanisms are discussed below.

Protein synthesis inhibitors

Interfering with macromolecules metabolism is likely to serve as the supporting mechanism in preventing cell growth. Such events usually begin at low peptide concentration before the rapid killing of bacteria due to perturbation of membrane at high peptide concentration. Notably, it is increasingly common to find protein synthesis-inhibiting AMPs having affinity against multiple targets. Several of these peptides have been studied in details. For example, The 13 residues 1HR1 is a synthetic variant of indolicidin (Friedrich *et al.*, 2001) which belongs to the cathelicidin family of polypeptides (Durr *et al.*, 2006). It is a polycationic, Trp-rich, and C-terminally amidated AMP isolated from the cytoplasmic granules of bovine neutrophils (Selsted *et*

al., 1992). Previous studies have documented that indolicidin and its synthetic analogues exhibited strong inhibitory effects against multiple bacterial, fungal, and viruses (Robinson *et al.*, 1998; Selsted *et al.*, 1992; Selsted *et al.*, 1993; van Abel *et al.*, 1995). Unlike AMPs that act upon the bacterial plasma membrane (Ganz & Lehrer, 1999; Lehrer *et al.*, 1989; Zasloff, 2002b), indolicidin has been shown to interact with bacterial nucleic acids to inhibit the cell replication or transcription processes leading to cell death (Subbalakshmi *et al.*, 1996). This unique mechanism of action is of great interest, as the ability to penetrate bacterial cell membrane and exert inhibitory effects on the intracellular targets can greatly avoid the problem of toxicity. Additionally, the indolicidin derivative CP10A exhibits multitude of inhibitory activities from membrane lysis to disruption of DNA, RNA, and protein synthesis (Friedrich *et al.*, 2001). CP10A had been shown to reduce the incorporations of histidine, uridine, and thymidine in *S. aureus* (Friedrich *et al.*, 2001). Apart from that, PR-39 interferes with the protein and DNA synthesis pathways to induces metabolic cessation (Boman *et al.*, 1993). Sublethal dose of pleurocidin, an AMP isolated from the skin secretions of winter flounder (Cole *et al.*, 1997), inhibited the growth of *E. coli* via protein synthesis arrest as observed with the reduction of histidine, uridine, and thymidine incorporations (Patrzykat *et al.*, 2002). It was only at lethal dose that pleurocidin becomes both bactericidal and inhibited macromolecular synthesis (Patrzykat *et al.*, 2002). Other AMPs such as dermaseptin and defensins also interrupt with protein synthesis (Jenssen *et al.*, 2006). Human neutrophil α-defensin induced sequential permeabilization of the outer and inner membrane of *E. coli* which was accompanied with failure of DNA, RNA, and protein synthesis (Lehrer *et al.*, 1989). However, loss of inner membrane integrity was found to be the key event associated with lethality. Therefore, multivalent membrane-active AMPs display high tendency to co-affect principally DNA synthesis thus suggesting the involvement of AMPs since the early translation steps in the protein expression system.

Nucleic acid synthesis inhibitors

Several AMPs are able to inhibit the production of DNA and RNA of the target organisms. Buforins are a class of DNA inhibitor first isolated in year 1996 from the stomach tissues of *Bufo bufo gargarizans* toad (Park *et al.*, 1996). Buforin I exhibited potent antimicrobial activity against fungi and wide range of gram-positive and gram-negative bacteria. Buforin II, a 21 amino acids fragment peptide derived from the enzymatic cleavage of buforin I by endoproteinase Lys-C displays stronger antimicrobial activity than the parent peptide (Park *et al.*, 1996). Later on it was clearly demonstrated by Park *et al*. that buforin II traverses the cell membrane and accumulates in the cytoplasm of *E. coli* (Park *et al.*, 2000). However, membrane leakage was not observed even at concentration five-fold above the MIC (Kobayashi *et al.*, 2000; Park *et al.*, 1998). Experiment using gel-retardation technique had detected high nucleic acid binding affinity of buforin II which strongly implies that buforin II inhibits cellular functions by targeting the DNA and RNA (Park *et al.*, 1998). The finding was supported by the notion that the parent AMP buforin I showed high sequence homology (37/39) to the N-terminal fragment of the DNA-binding protein histon H2A leading to the speculation that other AMPs which closely resemble H2A also exert their antimicrobial activity in a similar fashion as buforin II (Park *et al.*, 1998; Park *et al.*, 2000) such as parasin I and hipposin derived from the skin mucus of catfish (Birkemo *et al.*, 2003).

Even though both buforin II and other α-helical amphipathic AMPs are closely-related in terms of structural and physicochemical properties, the actual mode of actions can be varied considerably (Park *et al.*, 1998). Notably, the presence of proline hinge region in buforin II is critical for its transmembrane activity. Disruption or loss of hinge region hinders the peptide from entering the cell cytoplasm (Kobayashi *et al.*, 2000; Park *et al.*, 2000). In turn, these membrane-associated buforin II is able to induces

membrane permeation. This also suggests the functional duality in the actions of buforin II and how minor substitution in AMPs sequence produces novel antimicrobial activity.

The 13 amino acids indolicidin is a tryptophan-rich bovine neutrophil-derived AMPs belongs to the cathelicidin family of AMPs (Selsted *et al.*, 1992). The potent antimicrobial activity of indolicidin is attributable to permeabilization of plasma membrane while also possessing DNA inhibitory effects (Ahmad *et al.*, 1995; Ladokhin *et al.*, 1997; Schibli *et al.*, 2002). Interestingly, it was found that the membrane permeation activity was not accompanied with cell lysis (Falla *et al.*, 1996). Rather, *E. coli* treated with indolicidin showed filamentous appearance of cells together with a lack of thymidin uptake. Combining these observations, the mechanism of action of indolicidin is deduced to inhibit DNA synthesis exclusively but not RNA (Subbalakshmi & Sitaram, 1998). This finding was later supported by two study groups concluding the DNA-binding activity of indolicidin (Hsu *et al.*, 2005; Marchand *et al.*, 2006). Indolicidin targets specifically the abasic site of DNA and cross-links with the single- or double- stranded DNA (Marchand *et al.*, 2006). Hence, it was proposed that indolicidin probably interacts with the cell membrane causing membrane permeabilization at high concentration but not lysis to facilitate peptide entry into the cytoplasmic site without killing the bacteria (Hsu *et al.*, 2005).

Besides direct DNA binding, indolicidin also prevents DNA relaxation by inactivating the DNA topoisomerase 1 (Marchand *et al.*, 2006). Notably, this process occurs without DNA unwinding thus further suggesting the multiple inhibitory roles of indolicidin against various DNA-associated enzymes (Marchand *et al.*, 2006). The presence of homologous sequences in other DNA-binding proteins (e.g. DNA methylltransferase) further indicates that N-terminal fragment of indolicidin rather than the PWWP motif is responsible for its DNA-binding activity (Marchand *et al.*, 2006). Indolicidin had also been demonstrated to bind with high affinity against calmodulin in

a Ca^{2+}-dependent fashion (Sitaram *et al.*, 2003). Tachyplesin isolated from hemocytes of horseshoe crab *Tachypleus tridentatus* is another example of DNA inhibiting peptide (Nakamura *et al.*, 1988). It binds to the minor groove of DNA to exert inhibitory effect (Yonezawa *et al.*, 1992). Other AMPs such as PR-39 also exhibits nucleic acid inhibitory effects (Boman *et al.*, 1993).

Mitochondrion inhibitors

Histatin-5 is known for its antifungal activity against *Candida albicans*, the opportunistic fungus causing oropharyngeal candidiasis in most of the HIV-1-infected patients (de Repentigny *et al.*, 2004). It is a member of histatins, a group of histidine-rich cationic peptides present in the salivary secretions of human submandibular and parotid glands (Troxler *et al.*, 1990). Histatin-5 induced significant loss of mitochondrial transmembrane potential (Helmerhorst *et al.*, 1999) and generated reactive oxygen species (Helmerhorst *et al.*, 2001a) which caused damages to the treated *C. albicans* cells. The mechanism was demonstrated to begin with intracellular internalization of histatin 5 and then act upon the energized mitochondrion of the cell. Although histatin-5 does disrupt the cytoplasmic and mitochondrial membranes, the slow depolarization events reveal that histatin-5 is rather weak at the membrane level (Den Hertog *et al.*, 2004; Helmerhorst *et al.*, 1999; Ruissen *et al.*, 2001). This was suggested to be due to the poor amphipathic property of the peptide to initiates membranal insertion and pore formation (Helmerhorst *et al.*, 2001b; Raj *et al.*, 1990). Besides, other cellular targets may also be involved (Hancock & Diamond, 2000).

Microcins are a group of enterobacteria AMPs isolated mainly from *E. coli*. The microcins mcc25 which displayed potent antimicrobial activity against the closely-related bacteria such as *Shigella* and *Salmonella* was isolated from a faecal strain of *E. coli* (Salomon & Farias, 1992). As opposed to histatin-5, mcc25 is a mitochondrion

membrane-active peptide that inhibits the cytochrome c reductase (Niklison Chirou *et al.*, 2004). Mcc25 has been suggested as a potential antitumor agent as well (Niklison Chirou *et al.*, 2004).

Protein folding inhibitors

Chaperones are crucial for the proper folding of newly synthesized proteins (Ellis, 1990). Pyrrhocoricin, apidaecin, and drosocin are group of short (18 - 20) proline-rich insect-derived AMPs which inhibit bacterial chaperones, specifically the DnaK protein of *E. coli* but not GroEL which can be found in many bacteria including *E. coli* (Otvos *et al.*, 2000). Binding of these AMPs to DnaK is essentially stereo-specific (Otvos *et al.*, 2000). Such high specificities confer advantages in term of cell toxicity as they do not target the human equivalent chaperone heat shock protein 70. In addition, pyrrhocoricin and drosocin also prevent DnaK from refolding of misfolded proteins by inducing permanent closure of the peptide-binding cavity by the multihelical lid of DnaK (Kragol *et al.*, 2001).

Cell wall-targeting peptides

There are several ways in which AMPs affect cell wall synthesis and/or integrity. Mersacidin is a globular lantibiotic formed by the presence of four intramolecular thioether bridges (Prasch *et al.*, 1997). It is the smallest lantibiotic (20 amino acids, 1825 Da) known up to date and is neutral in charge. This peptide was originally expressed by *Bacillus* sp. strain HIL Y-85, 54728 which was not naturally competent (Chatterjee *et al.*, 1992). Later, the production of mersacidin was successfully established in the closely-related *Bacillus amyloliquefaciens* FZB42 via competence transformation (Herzner *et al.*, 2011). Mersacidin targets the peptidoglycan precursor lipid II to form a complex with the sugar phosphate head group (Brotz *et al.*, 1998a).

This impaired the transglycosylation step in cell wall biosynthesis (Brotz *et al.*, 1997). Since mersacidin targets the peptidoglycan biosynthesis pathway, it was found to attack particularly gram-positive bacteria such as methicillin-resistant *S. aureus* (MRSA) but not gram-negative or fungi (Chatterjee *et al.*, 1992; Hoffmann *et al.*, 2002). Cells treated with mersacidin were observed with accumulation of peptidoglycan precursors UDP-MurNAc-pentapeptide in the cytoplasm (Brotz *et al.*, 1997). By using a murine rhinitis model, mersacidin was able to eradicate MRSA colonization without stimulating inflammatory responses (Kruszewska *et al.*, 2004). Nisin is another example of lantibiotic that similarly targets the lipid II components for its antimicrobial activity (Brotz *et al.*, 1998b). Based on literature searches up to date, there was no PBPs-targeting AMPs being documented.

Staphylococcus epidermitis produces lantibiotic Pep5 which is characterized by the presence of three intramolecular rings (Sahl & Brandis, 1981). The peptide involves indirectly in cell wall lysis through the activation of cell wall-associated lytic enzymes (Bierbaum & Sahl, 1987). It has high binding affinity to the TAs and LTAs of gram-positive bacterial cell wall which competitively displaces the cell wall-associated amidases. This leads to premature release of the autolytic enzymes resulting in cell lysis and cell death. Together with another lantibiotic epidermin which is also produced by *S. epidermitis*, they have been proposed to have potential usage as novel catheters disinfecting agents (Fontana *et al.*, 2006).

Cell division inhibitors

A number of AMPs were demonstrated to have significant role in the process of septum formation during the bacterial cell division. As shown by Salomon and Farias, *E. coli* treated with mcc25 become significantly elongated without septation thus producing a filamentous morphology (Salomon & Farias, 1992). In addition, mcc25 acts

in a non-SOS-dependent mechanism hence suggesting its bacteriostatic mode of action (Salomon & Farias, 1992). AMPs-induced filamentation had also been described with indolicidin (Subbalakshmi & Sitaram, 1998), PR39 and the truncated variant PR26 (Shi *et al.*, 1994).

Lipopolysaccharide-binding peptides

Gram-negative bacteria have two distinct layers, the inner and the outer membrane. LPS constitutes the outer leaflet component of the outer membrane (Osborn, 1969). This structure is important in preventing and restricting the entry of harmful compounds into the cell. LPS molecule can be divided into three portions: the hydrophobic anchor Lipid A (LA) is a glucosamine-based phospholipids which constitutes the outer monolayer of LPS, the core polysaccharides, and the O-antigen repeats (Raetz *et al.*, 2007; Raetz & Whitfield, 2002). LPS or more specifically the LA molecule is well known as the bacterial endotoxin largely responsible for the toxicity and clinical manifestations associated with gram-negative bacterial infections. LPS can be shed off from the cell during the process of infection or the course of antimicrobial chemotherapy treatment (Tracey *et al.*, 1987). Endotoxin is detected by toll-like receptor 4 presented on the animal endothelial cells and immune cells including monocytes, neutrophils, macrophages, and dendritic cells (Akira *et al.*, 2006; Hoshino *et al.*, 1999; Poltorak *et al.*, 1998). This in turn stimulates the inflammatory mediators such as TNF-α and IL1-B (Beutler & Cerami, 1988; Dinarello, 1991) and activates co-stimulatory cells of the adaptive immune arm (Medzhitov & Janeway, 2000). However, in the case of endotoxemia where endotoxin is being released into the blood, overproduction of stimulatory molecules could lead to septic shock, severely damaging the blood vessels causing intravascular coagulations and eventually organ failures

(Bernard *et al.*, 2001; Esmon, 2000; van Deuren *et al.*, 2000). In the developing countries, endotoxemia represents a major cause of death (Parrillo, 1993).

In recent years, increasing interests have been grounded on the interactions between AMPs and bacterial LPS. Endotoxin-targeting AMPs have great potential to be developed as antiendotoxin or endotoxin-neutralizing agents to eliminate endotoxic shock in patients during or after treatment (Gough *et al.*, 1996; Hancock, 1999). AMPs binding to LPS are able to neutralize the endotoxin by preventing the overwhelming activation of inflammatory responses as a result of imbalance cytokines production (e.g. IL-1, TNF-α) mainly by the LPS-stimulated mononuclear and macrophage cells (Gee *et al.*, 2003; Kubo *et al.*, 2007; Mukhopadhyay *et al.*, 2004). Amphipathic and high proportion of cationic residues of AMPs have been suggested to be the principal binding motifs against the oppositely charged LA (Hoess *et al.*, 1993; Oren & Shai, 1998). Even for the relatively short ß-sheet AMPs, binding can still be formed between the ß-sheet cationic side chains and the anionic phosphates of LA (Frecer *et al.*, 2000). Muhle and Tam had successfully developed a novel ß-stranded cyclic AMP by mimicking the putative LPS-binding sites based on the LPS-binding protein family (Muhle & Tam, 2001).

1.6.3. Mechanism of bacterial AMPs resistance

Several reports have shown that resistance to AMPs might occur in certain microorganisms (McPhee *et al.*, 2003; Weidenmaier *et al.*, 2003). The reported mechanisms include D-alanylation of LTAs on cell surface, insertion of aminoarabinose to lipid A moiety of LPS, active efflux of AMPs via transporters, and enzymatic degradation of peptide chain (Kristian *et al.*, 2005; Nizet, 2006; Peschel, 2002).

The initiation of AMPs binding on bacterial membrane is primarily via electrostatic attraction forces. To interfere with the binding, the bacteria are able to

counteract by modification of the membrane chemical compositions leading to alteration of cell wall compositions and surface charges. In gram-positive bacteria, the cell wall is composed mainly of TAs and peptidoglycan. The cell wall-associated TAs are covalently linked to peptidoglycan and the membrane-associated TAs are anchored to cytoplasmic membrane via glycolipid linkage with its polyglycerophosphate (Gro-P) chain extending to the wall (Neuhaus & Baddiley, 2003). These negatively-charged TAs are the most abundant polyanions components of gram-positive bacteria which causes the accumulation of cationic AMPs on the anionic bacterial surface (Weidenmaier & Peschel, 2008). The bacteria are able to counteract by increasing the positive cell surface charges via D-alanylation of TAs and/or incorporation of L-lysine into phosphatidylglycerol which reduces the electrostatic attractions (Weidenmaier & Peschel, 2008). The former model represents the most described resistance mechanism against AMPs by the gram-positive bacteria. In fact, reduced electrostatic interaction with the cationic antibiotics gallidermin (Peschel *et al.*, 1999) and vancomycin (Peschel *et al.*, 2000) due to a decrease in the cell wall anionic property of *S. aureus* had been documented. D-alanylation of TAs involves the D-alanine-activating enzyme and a D-alanine-D-alanyl carrier protein ligase (Chevion *et al.*, 1974). These enzymes are encoded by genes on the dlt operon (Heaton & Neuhaus, 1992; Kristian *et al.*, 2005; Perego *et al.*, 1995). *S. pneumoniae* lacking dltA gene had been shown to be more susceptible to AMPs than the wild type strains (Kovacs *et al.*, 2006). The deletion of dlt operons in *S. aureus* (Peschel *et al.*, 1999), *Enterococcus faecalis* (Fabretti *et al.*, 2006), group A (Peschel *et al.*, 1999) and group B streptococci (Kristian *et al.*, 2005) as well as the probiotic strain *Lactobacillus rhamnosus* GG (Perea Velez *et al.*, 2007) demonstrated absence of D-alanyl esters of TAs and displayed enhanced susceptibility to AMPs. On the contrary, a recent study by Saar-Dover and colleagues found that reducing the anionicity of TAs did not contribute to AMPs resistance by decreasing the

membrane-bound AMPs in group B Streptococcus (Saar-Dover *et al.*, 2012). It was the conformational changes in D-alanylated TAs which resulted in increasing cell wall density that prohibited AMPs penetration through the cell wall (Saar-Dover *et al.*, 2012).

CPS acts principally by mean of exploitations of anionic molecules as the ionic decoy against the oppositely charged AMPs (Campos *et al.*, 2004; Llobet *et al.*, 2008; Spinosa *et al.*, 2007). Although the chemical compositions of CPS varied yet most of them are anionically charged. The action is comparable to that of chemokine decoy receptors whereby the production of decoy molecules by parasites and viruses are used to trap the chemokine hence preventing the activation of immune cells (Mantovani *et al.*, 2006). The presence of free CPS in the microenvironment surrounding the bacteria has significant role in the development of resistance and is of greater interest rather than the bound CPS. In fact, it had been demonstrated that encapsulated bacteria such as *K. pneumoniae* are able to shed free CPS to the extracellular complex (Straus *et al.*, 1985). For *S. pneumoniae*, competence-induced pneumococci cause cell-to-cell contact lysis of the competence-deficient cells by the CBP-bound lysins to release the CPS (Steinmoen *et al.*, 2002; Steinmoen *et al.*, 2003). UV-killed cells can similarly release the CPS from the bacterial cells (Llobet *et al.*, 2008).

The mechanism by which AMPs induce CPS release from bacterial surface had been suggested to occur in a way analogous to the mechanisms by which AMPs interrupt and release LPS (Vaara, 1992), Fresno *et al*. demonstrated that CPS anchorage to the cell surface of *K. pneumoniae* is mediated by electrostatic interaction between the CPS and LPS core region and further stabilized by divalent cations (Fresno *et al.*, 2006). The presence of AMPs interrupts with the ionic bridges formed between the neighboring CPS molecules which trigger the simultaneous release of CPS from the bacterial surface (Llobet *et al.*, 2008). The release of CPS was detectable at the

sublethal concentration and was inducible by the addition of AMPs (Llobet *et al.*, 2008) or by the activated neutrophils during the course of infections *in vivo* (Ganz, 1987; Selsted & Ouellette, 1995). As compared to the non-CPS mutant strains, the presence of CPS (both release naturally or AMPs-induced) can lead to dramatic reduction in the bacterial susceptibility against AMPs (Campos *et al.*, 2004; Llobet *et al.*, 2008; Spinosa *et al.*, 2007). Such mechanisms appear to reduce the killing efficacy by AMPs, resulting in delayed cell death hence allowing the bacteria to have more time to activate counter measures against AMPs. This leads to higher likelihood of developing secondary resistance mechanisms that are usually more time-consuming such as membrane charge alterations.

Nevertheless, the actual mode of AMPs resistance by the bacteria is not completely clear and there are exceptions to the observations. For example, both wild-type and CPS-deficient mutant of *Campylobacter jejuni* were as resistant to AMPs (Zilbauer *et al.*, 2005). Also, *S. pneumoniae* of serotypes 1, 2, and 4 were more sensitive to AMPs than the isogenic CPS mutants (Beiter *et al.*, 2008). One possible explanation to this is that the presence of CPS might in turn mask the surface modifications produced by the pneumococci in an attempt to reduce the susceptibility against AMPs (Beiter *et al.*, 2008). In a recent study, serotype alone was found to be insufficient to deduce the susceptibility of *S. pneumoniae* against AMPs but rather the differences in susceptibility was suggested to be due to genetic variation of the individual pneumococcal strains (Habets *et al.*, 2012). Hence, the roles of CPS as well as other molecules in enhancing AMPs resistance are debatable and more findings are needed to support the notion.

On the other hand, certain bacteria are able to secrete proteolytic enzymes to inactivate the microbicidal activity of AMPs. This had been determined in *S. aureus* whereby the release of aureolysin inactivated LL-37 and hence the bacteria resistance

against the clearance mechanism by the host innate immunity (Sieprawska-Lupa *et al.*, 2004).

1.7. AMPs as novel antimicrobial agents

The development and the eventual availability of novel antibiotics has never been in pace with the rate at which the bacteria adapt and develop resistant. This is of great concern as only very few new family of antibiotics have been discovered in the past 30 years (Hancock, 2001). Conventional antibiotics have observed considerable decline in antimicrobial efficacy due to the progressive expansion of antibiotic-resistant organisms and increasing tolerability against the commonly used antibiotics (Davies, 1994; Schutze *et al.*, 1994). With the reports of not only PISP and PSSP but also the very-high-level resistant strains, novel classes of antibiotics active against pneumococcus are extremely in demand in the near future (Ganz, 2001; Schrag *et al.*, 2004). For this reason, the discovery of AMPs and its potential application as novel therapeutic agents sheds light on this problem (Hancock & Sahl, 2006; Sang & Blecha, 2008).

AMPs are rapid acting molecules whereby the killing activity begins instantaneously upon exposure of the cells to the AMPs. These direct and rapid modes of bacterial destruction greatly reduce the chances of resistance development by the bacteria. Indeed, even after such a long time of evolutionary, the continue survival of host indicates that the harmful microbes do not seem to acquire sufficient resistance against AMPs. Many studies have thus investigated into the potential use of AMPs as novel antibacterial agents against new targets (Boman, 1995; Devine & Hancock, 2002; Lehrer & Ganz, 1999; Zasloff, 2002a).

From isolation of AMPs from natural sources and determination of their biological activity, researchers are investing intense efforts into developing synthetic analogues of AMPs with enhanced antimicrobial activity while reducing cell toxicity

effects to be developed as a clinically usable therapeutic agents. In fact, AMPs with multifunctional roles have been increasingly identified ever since. One good example is the continued expansion of the entries in various AMPs databases, particularly the Antimicrobial Peptide database which initially included only the antibacterial, antiparasital, and antifungal peptides but has then expanded to include antiviral, insecticidal, anti-HIV, antiprotist, spermicidal, as well as anticancer/antitumor peptides and AMPs with chemotactic activity (Wang *et al.*, 2009). The design of novel synthetic analogues are mainly based on existing AMPs isolated from various sources and to which determined with strong antimicrobial activities.

1.7.1. Cathelicidins

Human polymorphonuclear neutrophilic leukocytes produce a wide variety of AMPs which are mainly localized in the granules (Giovannini *et al.*, 1987) (Levy, 1996). Among them, cathelicidins family of AMPs is predominantly found in the peroxidase negative granules of vertebrate neutrophils (Zaiou & Gallo, 2002; Zanetti *et al.*, 1995). These peptides represent an important effector component in human innate immunity (Zanetti, 2004). Despite showing considerable structural variations, cathelicidins as well as other amphipathic α-helical AMPs including IsCT, Magainins, Cecropins, LL-37, Ovispirins, Melittin, Pleurocidin, and SMAP29 are generally cationic, linear, and has no cysteine.

The C-terminal fragment of cathelicidins is widely recognized as the biologically active domain responsible for the antimicrobial activity of cathelicidins. Unlike the C-terminus which possess highly varied amino acid sequences and secondary structure, the 12 kDa N-terminus of cathelicidins known as cathelin is highly conserved. Cathelin has been found to possess dual-functionality in coordinating the cystein-proteinase-

mediated tissue damage and also inhibits the growth of *E. coli* and MRSA (Zaiou *et al.*, 2003).

Despite large number of cathelicidins being isolated from porcine and bovine neutrophils, to date the only cathelicidin identified in human is the human cationic antimicrobial protein hCAP18 (Agerberth *et al.*, 1995; Cowland *et al.*, 1995; Gudmundsson *et al.*, 1996; Larrick *et al.*, 1995; Larrick *et al.*, 1996). hCAP18 was first isolated from human bone marrow cells and testis (Agerberth *et al.*, 1995). Later, hCAP18 had also been detected from various immune and epithelial cells (Frohm Nilsson *et al.*, 1999) (Agerberth *et al.*, 1999; Bals *et al.*, 1998) (Frohm *et al.*, 1997) (Malm *et al.*, 2000) (Di Nardo *et al.*, 2003). The C-terminus of hCAP18 is known as LL-37, a 37 amino acids peptide led by two leucine residues (De Smet & Contreras, 2005). This short peptide had been detected in bronchial epithelial cells, bronchial glands, and alveolar macrophages (Agerberth *et al.*, 1999; Bals *et al.*, 1998). LL-37 is known for its indispensable role in pulmonary innate immunity (Zhang *et al.*, 2000b). Moreover, LL-37 exhibits broad spectrum antibacterial activity (Turner *et al.*, 1998) and the effect can be further synergized with the presence of defensins (Nagaoka *et al.*, 2000). Additionally, LL-37 serves important roles in wound healing and inflammatory responses by functioning as re-epithelialization promoting agent (Heilborn *et al.*, 2003), as chemotactical agent to recruits T cells, monocytes, and neutrophils to the site of infections (De *et al.*, 2000), and roles in angiogenesis and arteriogenesis (Heilborn *et al.*, 2003; Koczulla *et al.*, 2003). LL-37 also possesses endotoxin-neutralizing effects (Golec, 2007; Nagaoka *et al.*, 2001). Deficiency in LL-37 has been suggested to be associated with specific clinical conditions as seen with the manifestation of periodontitis in morbus Kostmann patients (Putsep *et al.*, 2002). A significant drawback observed with LL-37 is the associated cytotoxicity against mammalian cells (Johansson *et al.*, 1998).

1.7.2. Magainins

Amphibian skin glands secrete a variety of AMPs as a measure to protect the constantly moisturized skin where essential metabolic processes are taken place from microbial infections (Barra & Simmaco, 1995; Rollins-Smith *et al.*, 2005). Magainins are produced naturally by the skin gland of African clawed frog *Xenopus laevis* (Zasloff, 1987). Magainins are α-helical cationic peptides that exhibit broad spectrum antibacterial activity against gram-positive and gram-negative bacteria, antifungal, and antiprotozoan activities while having low toxicity against eukaryotic cells (Bevins & Zasloff, 1990; Zasloff, 1987). Similar to other frog-skin derived peptides, magainins also have important roles in the regulation of mammalian hormones and neurotransmitters (Bevins & Zasloff, 1990). Magainin 1 and magainin 2 are 23 amino acids in length and differ by two residual substitutions between them (Zasloff, 1987). Magainin 2 is a membrane active peptide capable of forming transmembrane pores in the bacterial cell membrane leading to cell lysis (Kobayashi *et al.*, 2000; Park *et al.*, 1998; Park *et al.*, 2000; Wenk & Seelig, 1998). A schematic illustration of transmembrane pores formed by magainins is shown in figure 1.7 (Yang *et al.*, 2000).

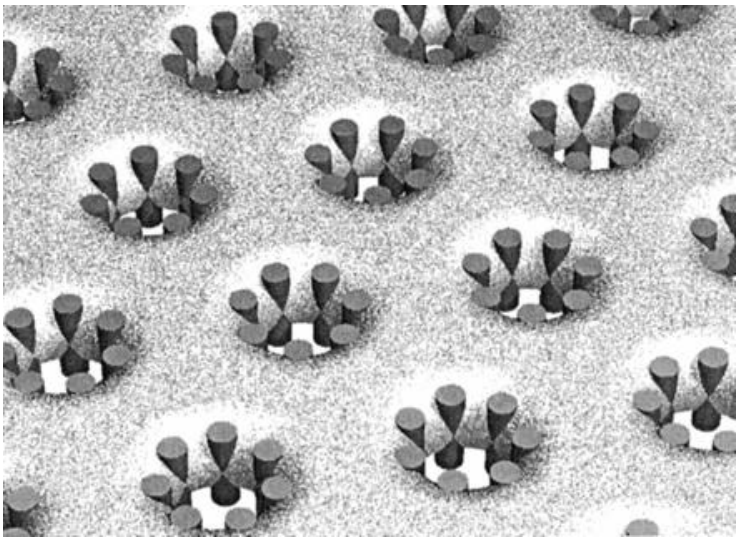


Figure 1.7: Schematic representation of formation of magainin pores in membrane, inducing cellular leakage and cell death (adapted from Yang *et al.*, 2000).

Another subtype, peptidyl-glyclyleucine-carboxyamide (PGLa) is a C-terminally amidated 21 amino acids peptide with sequence GMASKAGAIAGKIAKVALKAL (Andreu *et al.*, 1985; Hoffmann *et al.*, 1983). Interestingly, magainin 2 and PGLa are able to interact cooperatively in a synergistic manner against bacteria, artificial membranes, and also tumor cells (Matsuzaki *et al.*, 1998). Magainin 2 is a slow pore former but the pores formed remain stable and long-lived. In contrast, PGLa is a rapid pore former but the pores formed are rather unstable as compared to magainin 2. When the peptides are presented to membranes in 1:1 ratio, the complex formed display the advantageous properties of the respective peptide producing fast pore formation rate with moderate stability. These properties warrant their potential use as new antibiotic agents (Matsuzaki, 1998).

Apart from magainin and PGLa as described above, several other classes of amphibian AMPs have also been reported. This includes brevinins from the European frog *Rana brevipoda porsa* (Morikawa *et al.*, 1992), temporins from *R. temporaria* (Simmaco *et al.*, 1996), dermaseptin from *Phyllomedusa sauvagii* (Mor *et al.*, 1991), and Ranalexin from *R. catesbeiana* (Clark *et al.*, 1994). Recently, a novel class of frog AMPs has been isolated from the skin of *Amolops loloensis* in southwest China (Wang *et al.*, 2008) and also the first documentation of sea frog *R. cancrivora* AMPs (Lu *et al.*, 2008). For these reasons, it was speculated that amphibians skin as well as other organisms in the nature might own a large pool of novel AMPs that is yet to be discovered for potential therapeutic applications (Barra & Simmaco, 1995).

1.7.3. Prophenins

Prophenins (PF) are group of AMPs isolated from porcine leukocytes. Prophenin-1 (PF-1) is a proline- (53.2%) and phenylalanine-rich peptide (19.0%) having 79 residues encoded on a cathelin-containing precursor (Harwig *et al.*, 1995a). The 60

residues fragment at the N-terminus consists of two sets of identical and near-identical decamer repeats FPPPNFPGPR. Later, two major PF-2 variants prophenin-2-pyroglutamic acid (PF-2-pyr) and prophenin-2-glutamine (PF-2-gln) had also been detected from the porcine pulmonary tissue extracts (Wang *et al.*, 1999). PF-2-gyr is 80 amino acids in length with an N-terminal pyroglutamic residue whereas the PF-2-gln consists of an amidated N-terminal glutamine (Wang *et al.*, 1999; Wang *et al.*, 2000). The level of PF-1 is higher in leukocytes as compared to the pulmonary tissue extracts (Wang *et al.*, 2000). In addition, shorter peptides corresponding to the N-terminal 17- and 18- residues fragment of PF have also been reported. The 18 residues peptide showed comparatively higher antibacterial activity against the gram-positive *Bacillus megatarium* Bm11 than *E. coli* D21 in the absence of salt (Wang *et al.*, 1999). Surfactant containing AMPs, especially the 18 residues peptide has been suggested as a potential local treatment agent for pulmonary infections (Wang *et al.*, 2004).

1.7.4. Defensins

Defensins are one of the two major genetic categories of mammalian AMPs beside cathelicidins. Ganz *et al*. had first identified human defensins from the neutrophils (Ganz *et al.*, 1985). The mature defensins peptides shared several physicochemical properties including short peptide length of 18 to 45 amino acids, net cationic charge (+1 to +11), lack of acyl or glycosyl-chain modifications, has six conserved cystein residues, three intramolecular disulfide bridges, and turn-linked β-strands dominated tertiary structures (Kagan *et al.*, 1994; Lehrer *et al.*, 1993). Upon ingestion of invading bacteria by the immune cells, defensins in the phagocytic vacuoles will be delivered to the bacterial cells to induce immediate killing (Andreu & Rivas, 1998), principally by targeting the plasma membrane (Liu *et al.*, 2008a; Papo & Shai, 2003). Defensins also modulate the intracellular signaling events, stimulating the

maturation of host defense leucocytes and exhibit multiple antiinfective activities (Boman, 1995; Xiong *et al.*, 1999).

Both α- and β-defensins are genetically and structurally more closely related as compared to θ-defensins (Selsted *et al.*, 1993). They are distinguishable by the linear spacing and presence of tridisulfide bridges formed by six conserved cysteine residues in the peptide chain (Kagan *et al.*, 1994; Lehrer *et al.*, 1993). The disulfide bridges are suggested to be associated with antimicrobial activity (Daher *et al.*, 1986; Mandal & Nagaraj, 2002). The N-terminally truncated human β-defensins 1 (hBD-1) isoform is of interest due to its strong antibacterial effects (Valore *et al.*, 1998). Following that, two additional forms, hBD-2 and hBD-3 have been detected from the scales of a psoriatic skin patient (Harder *et al.*, 1997, 2001). In skin diseases such as psoriasis (Harder & Schroder, 2005) and in bronchoalveolar inflammation (Hiratsuka *et al.*, 2003; Ross *et al.*, 2004), the concentrations of hBD-2 and hBD-3 were found to increase significantly. In a study conducted by Lee *et al.*, lysozyme and β-defensin-2 were found to be interacting in a synergistic manner against *S. pneumoniae* (Lee *et al.*, 2004a). Although the fourth type, hBD-4 has not yet been isolated from any natural source, the sequence based on cDNA analysis had been predicted and synthesized artificially (Garcia *et al.*, 2001).

A number of studies have documented the importance and potential role of defensins in diseases and microbial infections. The concomitant lower level of hBD-2, hBD-3 due to T-helper type 2 cytokine upregulation (Nomura *et al.*, 2003) had greatly increased the risks of skin infections by bacteria, fungi, or viruses among patients with atopic dermatitis (Ong *et al.*, 2002). In the event of psoriatic lesions, patients suffer unexpectedly low episodes of infections (Harder & Schroder, 2005). Moreover, the enteric α-defensin subclass is particularly vital in maintaining the equilibrium of microbiota in the intestinal mucosal (Salzman *et al.*, 2010). Deficiency in α-defensin

had been associated with ileal Crohn's disease and enteric infections (Wehkamp *et al.*, 2005; Wilson *et al.*, 1999). *In vivo* studies using mice challenged by enteric *Salmonella typhimurium* indicated that the tight regulation on the production of defensins as well as other AMPs are critical as aberrant expression of enteric α-defensin can alter the composition of commensal microbiota considerably (Salzman *et al.*, 2010). Interestingly, the role of β-defensin in sperm maturation had also been described (Yudin *et al.*, 2003; Zhou *et al.*, 2004).

1.7.5. Protegrins

Protegrins are membrane-active peptides act by forming weakly-anionic selective channel in the bacterial membrane (Lai *et al.*, 2006; Mani *et al.*, 2005; Sokolov *et al.*, 1999). Members from protegrins family of AMPs share the physicochemical properties of 16 to 18 amino acids in length, cationic, arginine- and cysteine-rich, and adopt a secondary structure of two-stranded antiparallel ß-sheet structure (Fahrner *et al.*, 1996; Kobayashi *et al.*, 2005; Sokolov *et al.*, 1999). Amphiphilicity, charge, and the native peptide structure determine the antibacterial activity of protegrins (Chen *et al.*, 2000). The overall structure maintains primarily by the bidisulphide bridges and also hydrogen bonding is crucial for antibacterial activity of protegrins rather than the presence of specific residues or its stereotype (Chen *et al.*, 2000). It has been suggested that LPS-targeting represents the principal mode of action by protegrins as protegrins showed higher affinity against gram-negative than gram-positive bacteria (Chen *et al.*, 2000). To date, as much as five highly homologous native protegrin (PG-1 to 5) had been identified (Zhao *et al.*, 1995; Zhao *et al.*, 1994). They exhibit broad spectrum antimicrobial activity against a number of common pathogens such as *E. coli, Candida albicans, Neisseria gonorrhoeae, and Listeria monocytogenes* (Kokryakov *et al.*, 1993; Qu *et al.*, 1996; Tamamura *et al.*, 1995; Yasin *et al.*, 1996). In addition, the antiviral

activity against HIV had also been documented (Tamamura *et al.*, 1995). The promising outcomes from the use of protegrins especially in oral mucositis (Bellm *et al.*, 2000; Mosca *et al.*, 2000) and respiratory-associated diseases (Cole & Waring, 2002) warrant further development of protegrins as novel therapeutic agents.

1.8. Designing novel antimicrobial peptides

Various strategies in designing novel synthetic analogues of AMPs have been described. One widely used method is the sequence-based approach which correlates antimicrobial activity as the function of change with regards to physicochemical properties of AMPs. This approach is more direct and simple in design and does not usually require high level computer modeling. Ueno *et al*. had attempted to design novel cationic AMPs based on a conservative strategy via acid-amide substitution (i.e. Glu to Gln, Asp to Asn) (Ueno *et al.*, 2011). These substitutions will not cause major conformational alteration as compared to the parent peptide thus helps preserve the integrity of the original structure to the highest extent (Ueno *et al.*, 2011). Interestingly, the newly generated peptides NP1P, NP2P, and NP3P showed marked increased in antibacterial activity against the gram-positive *S. aureus, Bacillus subtilis,* and *Micrococcus luteus* as well as the gram-negative *P. aeruginosa, S. typhimurium*, *E. coli*, and *Serratia marcescens* (Ueno *et al.*, 2011). On the other hand, Pasupuleti *et al*. noticed that end-tagging AMPs with hydrophobic amino acids Trp or Phe enhanced antimicrobial activity against both *S. aureus* and *E. coli*. The high potency of peptide was suggested to be due to high bacterial binding which caused bacterial cell lysis (Ueno *et al.*, 2011). On top of this, several of the novel synthetic AMPs designed based on considerations on four physicochemical parameters including NetC, hydrophobicity, hydrophobic moment, and polar angle were found to exhibited high selectivity against *Vibrio* spp. (Chou *et al.*, 2008).

Great majority of natural AMPs were characterized by the cationicity window between NetC of +4 to +6 (Giangaspero *et al.*, 2001), which appears that AMPs would have an optimal working window for best antimicrobial activity (Tossi *et al.*, 2000). Dathe *et al*. demonstrated that increasing the NetC of magainins 2 up to the threshold +5 enhanced antimicrobial activity of the peptides (Dathe *et al.*, 2001). However, no additional effect was noted up to a NetC of +7 but hemolytic activity become gradually significant (Dathe *et al.*, 2001). Increased in NetC of AMPs at the polar face has been suggested to enhance antimicrobial activity of the peptides. This is evident where single amino acid substitution from serine to lysine at the polar face of S16 produced K11 with two-fold increments in therapeutic index (Jin-Jiang *et al.*, 2012). A study conducted by Jiang *et al*. showed that both NetC and number of cationic residues on polar face of peptides were associated with antimicrobial activity and hemolytic activity of α-helical AMPs (Jiang *et al.*, 2008). The antimicrobial potency of the peptides correlated well with NetC of peptides within the window of +4 to +8 with minimal hemolytic activity; lowering than +4 rendered the peptides ineffective while overly high positive charges (≥ +9) was associated with high level hemolytic activity (Dathe *et al.*, 2001).

The influence of peptide hydrophobicity on α-helical AMPs was investigated by Chen *et al*. in a study using $V13K_L$ as the lead peptide (Chen *et al.*, 2007). Hydrophobicity of the peptides was altered systematically by alternatively substituting leucine with alanine. It was found that high antimicrobial activity of peptide fell within an optimum hydrophobicity window. Deviation from the specific range would render the loss of antimicrobial activity with the peptides (Chen *et al.*, 2007). Moreover, high hydrophobicity and amphipathicity were associated with hemolytic activity (Chen *et al.*, 2007; Chou *et al.*, 2008). Yan *et al*. extracted the 15 residues C-terminal segment of melittin and systematically altered the hydrophobicity of the peptides (Yan *et al.*, 2003). It was found that increased in hydrophobicity but not amphipathicity enhanced

antimicrobial activity although the effects at specific positions were more significant than others (Yan *et al.*, 2003). Interestingly, the antimicrobial and hemolytic activities were suggested to favor the opposite faces and this indicates that antimicrobial activtity could be dissociated from the side effects with careful consideration on region/face of the peptides (Yan *et al.*, 2003).

The presence of specific amino acid residue is sometime crucial for the antimicrobial activity of the peptides. Huang *et al*. noticed that substitution of the nonpolar face amino acid leucine which located at the 9^{th} position from the N-terminus of S16 with a polar amino acid (arginine, serine, or lysine) would cause a dramatic reduction in the antimicrobial potency of the peptide by more than sixteen-fold (S16 MIC = 3.125 µg/ml, S9 MIC = 50 µg/ml, K9 and R9 MIC = > 50 µg/ml) (Huang, 2000). Indolicidin is an AMP from bovine neutrophils with high proportion of tryptophan and proline residues (ILPWKWPWWPWRR-NH_2) (Selsted *et al.*, 1992). Subbalakshmi and colleagues have investigated into the roles of these residues in the sequence in relation to antimicrobial and hemolytic activity (Subbalakshmi *et al.*, 1996). It was determined that the presence of tryptophan contributed to the hemolytic activity of the peptide and the analogue with tryptophan replaced by phenylalanine displayed no hemolytic activity while having comparable antimicrobial activity (Subbalakshmi *et al.*, 1996). On the other hand, Loose *et al*. undertaken a unique linguistic approach for the design of AMPs by looking at the sequence of amino acids as "grammar" of a formal language (Loose *et al.*, 2006). The basis of the linguistic model is related to repeated occurrences of sequence modules which resembles phrases of a language (Loose *et al.*, 2006). Set of regular grammars were constructed to describe this language. In the example given by the authors, the sequence QxEAGxLxKxxK (where "x" denotes random amino acid) is highly common among cecropins, the insect AMPs (Loose *et al.*, 2006).

C-terminal amidation also represents a potential strategy to enhance the antibacterial potency of peptides and to increase the proteolytic resistibility of the peptides (Hou *et al.*, 2011). A C-terminally amidated thanatin which is an insect-derived *Podisus maculiventris* showed strong antibacterial effect against gram-negative bacteria, in particular, the extended-spectrum B-lactamase-producing *E. coli*. (Fehlbaum *et al.*, 1996; Hou *et al.*, 2011).

1.9. Justification and objective of the study

Although the first PCV7 vaccine has been available since year 2000, however, none of the PCVs are currently included under the childhood vaccination scheme in Malaysia. Documentations on the serotypes distribution among the Malaysian population is especially scarce and only a few studies being reported throughout the years (Le *et al.*, 2012). Continued monitoring of serotype distribution is important to predict the potential coverage of PCVs among the local population and to recommend on the use of PCV for routine immunization. The prevalence of penicillin-susceptibility, invasiveness, and age groups of patients can be obtained as well.

Pneumococcal resistance to conventional antibiotics especially β-lactam antibiotics has been increasingly reported worldwide. Novel antimicrobial agents are exceptionally in demand. Owing to its rapid and broad spectrum antibacterial activity, AMPs represents the most potential next-generation antimicrobial candidates. The hypothesis of the current study was synthetically designed novel antimicrobial peptides is potentially more effective in their antimicrobial activity and cell cytotoxicity than presently available antimicrobial agents. The potential therapeutic efficacies of the designed AMPs were assessed using *in vivo* murine pneumococcal infection models. Thus, the study was designed with the following objectives:

1. To determine the antibiotic susceptibility and serotype distribution of *S. pneumoniae* among the Malaysian population.
2. To design synthetic antimicrobial peptides with high reactivity against penicillin-resistant *S. pneumoniae*.
3. To assess the *in vitro* antibacterial activity and cell toxicity of synthetic antimicrobial peptides.
4. To develop mouse models of lethal pneumococcal infections for the determination of the suitable mode of administration of these synthetic antimicrobial peptides *in vivo*.
5. To determine the toxicity, therapeutic efficacy and therapeutic synergism of synthetic antimicrobial peptides *in vivo*.

CHAPTER 2

MATERIALS & METHODS

2.1. Isolates collection and identification

A total of 151 pneumococcal isolates were obtained from clinical samples which were processed and stored at the Microbiology Laboratory of the University of Malaya Medical Centre (UMMC), Malaysia from March 1999 to February 2007 (except year 2001 and 2004). Both invasive and noninvasive isolates from patients of all ages were included. The source of the isolates included blood, cerebrospinal fluid (CSF), nasopharyngeal (NP) secretion, tracheal secretion, sputum, bronchoalveolar lavage (BAL), and others. The isolates were grown on 5% (v/v) horse blood agar at 37°C under 5% CO_2 for 12 - 15 hours before being subjected to biochemical identification tests and molecular assays.

2.1.1. Hemolytic pattern on blood agar

S. pneumoniae isolates were grown overnight on defibrinated sheep blood agar (Oxoid, UK) at 37^{o}C under 5% CO_2. The bacteria produced α-hemolysis resulting in greenish-brown appearance of the agar (Figure 2.1). This is due to the incomplete breakdown of hemoglobin to methemoglobin by hydrogen peroxide of *S. pneumoniae.*

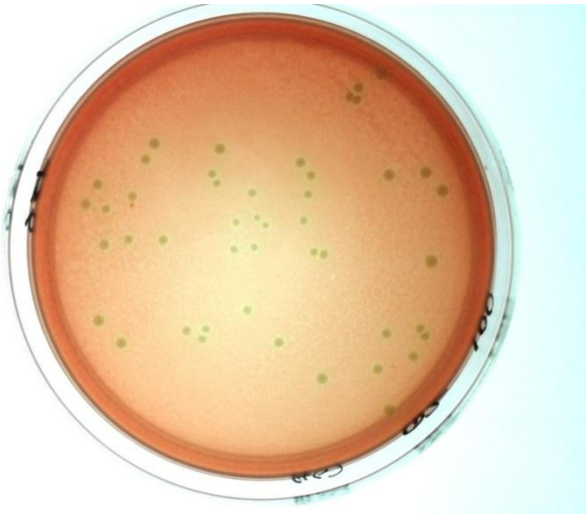


Figure 2.1: α-hemolysis by *S. pneumoniae* producing greenish-brown agar area surrounding the colonies.

2.1.2. Gram stain

Three to four well isolated pneumococcal colonies were directly suspended in phosphate buffered saline (PBS) on a clean glass slide and heat-fixed. The slide was then flooded with the primary stain crystal violet for 1 min, washed with distilled water, flooded with the mordant Lugol's iodine for 1 min, washed with distilled water, added with few drops of 70% (v/v) ethanol for rapid decolorization and again washed with distilled water before addition of diluted carbol fuschin for 1 min. The slide was washed with distilled water and air-dried for viewing using a light microscope. *S. pneumoniae* was observed as gram-positive in single, diplococci, or in long chain form (Figure 2.2).

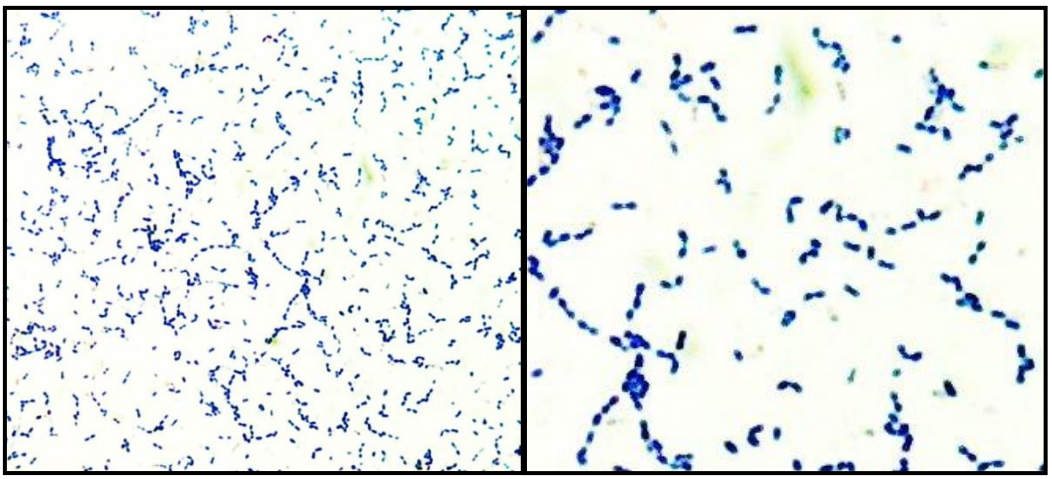


Figure 2.2: Gram positive staining of the overview (left) and close-in view (right) of *S. pneumoniae* at 1000X magnification.

2.1.3. Catalase test

Three to four colonies from an overnight culture were picked using a wooden applicator stick and dipped onto a glass slide with two to three drops of 3% hydrogen peroxide. Observation of bubbling (oxygen) was positive reaction which indicated the production of catalase enzyme in the bacteria. *S. pneumoniae* is catalase-negative and can be differentiated from the catalase-positive *Staphylococcus* spp..

2.1.4. Ethylhydrocupreine hydrochloride (optochin) susceptibility test

Overnight culture of the isolate on sheep blood agar was adjusted to OD_{625} 0.08 – 0.1 in PBS. A lawn of the culture was then made on a fresh sheep blood agar and the ethylhydrocupreine hydrochloride (optochin) disk (Oxoid, UK) was placed at the centre of the lawn before incubation at 37°C under 5% CO_2 for 18 - 24 hrs (Figure 2.3). *S. pneumoniae* is susceptible and produces an inhibition zone of ≥ 14mm in diameter according to the Clinical and Laboratory Standard Institute (CLSI) guidelines which is distinguishable from the closely-related viridans streptococci.

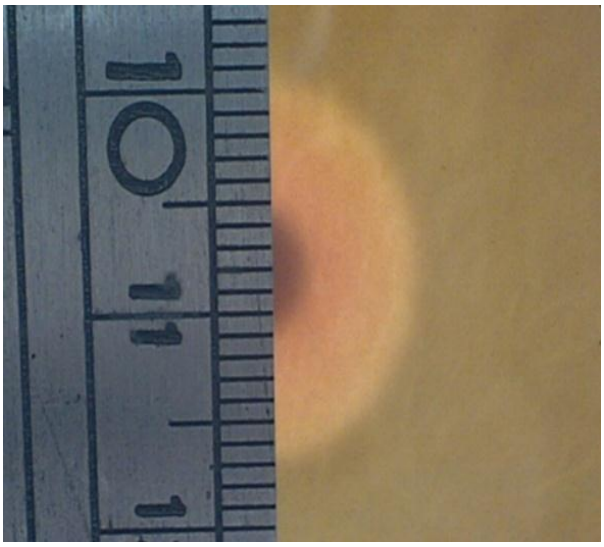


Figure 2.3: *S. pneumoniae* produced ≥ 14 mm zone of inhibition with optochin disk test.

2.1.5. Bile solubility

S. pneumoniae is susceptible to bile lysis. A few drops of 10% (w/v) sodium deoxycholate were added into the bacteria suspended in Mueller-Hinton Broth (MHB) and incubated at 37°C for 10 mins. The turbidity of the suspension was cleared as compared to the non-bile soluble *Streptococcus mitis* (Figure 2.4).

Figure 2.4: *S. pneumoniae* was cleared by bile lysis (right) as compared to *S. mitis* (left).

2.2. Antibiotic susceptibility testing

The penicillin susceptibility of *S. pneumoniae* was determined using the agar dilution method according to the CLSI guidelines (Clinical and Laboratory Standards Institute, 2005). Penicillin in powder form (Sigma Chemical Co., St. Louis, Mo) was dissolved in sterile deionized distilled water as recommended by CLSI according to the formula:

$$W = \frac{V \times C}{p}$$

where W is the weight of antibiotic powder (g); V is the volume of solvent; ρ is the potency of the antibiotic; and C is the desired concentration of the antibiotic to be prepared. The volume of the solvent was adjusted according to the weight of antibiotic weighed to ease and allow a more accurate preparation of stock concentration. The stock antibiotic prepared was then filter sterilized using a 0.20 µm disposable syringe filter. The stock antibiotic was then diluted to an appropriate working concentration

followed by two-fold serial dilutions of the antibiotic in sterile deionized distilled water in multiple vials. This was to avoid repeated freeze-thawed cycle that would reduce the potency of antibiotics. The stock and working solutions prepared were stored in -20^{o}C.

The penicillin susceptibility tests were conducted on Mueller Hinton Agar (MHA) (Oxoid, UK) containing 5% (v/v) defibrinated sheep blood (Oxoid, UK) supplemented with penicillin. All growths were incubated at 37°C under 5% CO_2 for 18 – 24 hrs. The MHA was prepared in distilled water according to manufacturer's protocol and autoclaved under standard condition (121^{o}C, 15 mins). The molten agar was then equilibrated to 55^{o}C in a waterbath. Each petri dish was first added with 2 ml of the antibiotic at the respective desired working concentration and 1 ml of defibrinated sheep blood (Oxoid, UK) to 5% (v/v) final concentration. Subsequently, 17 ml of the molten agar was poured into the petri dishes and gently swirled immediately to ensure even distribution of the antibiotic in the agar. The plates were allowed to solidify at room temperature before storage at 4^{o}C in refrigerator until use. *S. pneumoniae* ATCC 49619 was included in each run as quality control strain. Non-penicillin containing blood agar plates were also prepared as control in each run.

2.3. Multiplex Polymerase Chain Reaction (PCR) serotyping

2.3.1. DNA extraction

Pneumococcal DNA extraction was performed as described previously (Unal *et al.*, 1992). Briefly, bacterial colonies were suspended in 15 µl of distilled water containing 50 µg/ml lysostaphin and incubated at 37°C for 10 mins. Bacterial colonies suspended in 15 µl of distilled water were treated with 10 µg/ml Proteinase K and 0.l mM Tris HCl pH 7.5 and incubated at 37°C for 10 mins. Subsequently, the suspension was boiled for 5 mins and finally centrifuged at 13000 rpm for 2 mins.

The supernatant obtained was used as the template in the multiplex polymerase chain (PCR) reaction.

2.3.2. Multiplex PCR scheme

The primers used in this study were extracted from previously published sequences and the PCR protocol was as previously described (Pai *et al.*, 2006). Briefly, the primers were grouped into seven multiplex reactions as shown in Figure 2.5. Each reaction was designed to include four primer pairs targeting four different serotypes/serogroups and another primer pair *cps*A targeting the common region of the cps operon as internal positive control. Control strains of known serotypes by Quellung reaction representing different serotypes and serogroups were included as positive control in the respective serotyping pools. The serotypes included 1, 2, 3, 4, 5, 8, 13, 14, 20, 21, 31, 34, 37, 38, 39, 40, 44, 46, 6A, 7A, 7B, 7C, 7F, 9A, 9N, 9L, 10A, 10F, 11A, 11D, 11F, 12A, 12B, 12F, ISA, 15B, 15C, 15F, 16A, 16F, 17F, 33A, 35B, 35F, 35A, 35C, and 47F. The optimal PCR condition for a 25µl reaction included 1X PCR buffer (Fermentas, Lithuania), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 2U Taq Polymerase (Fermentas, Lithuania) and primer pairs at varying concentrations as shown in Table 2.1 (Pai *et al.*, 2006). The PCR cycling was carried out in an Eppendorf Gradient Mastercycler with initial denaturation step at 94°C for 4 mins and the 30 amplification cycles were performed with denaturation at 94°C for 45 s, annealing temperature at 54°C for 45 s and extension temperature at 65°C for 2 mins and 30 s and finally completed with an extension at 72°C for 2 mins (Table 2.2). The PCR product was electrophoresed on a 2% (w/v) Tris-acetic EDTA (TAE) agarose gel (Fermentas, Lithuania) for 1 hour at 70V and the bands were analyzed using a UV transilluminator.

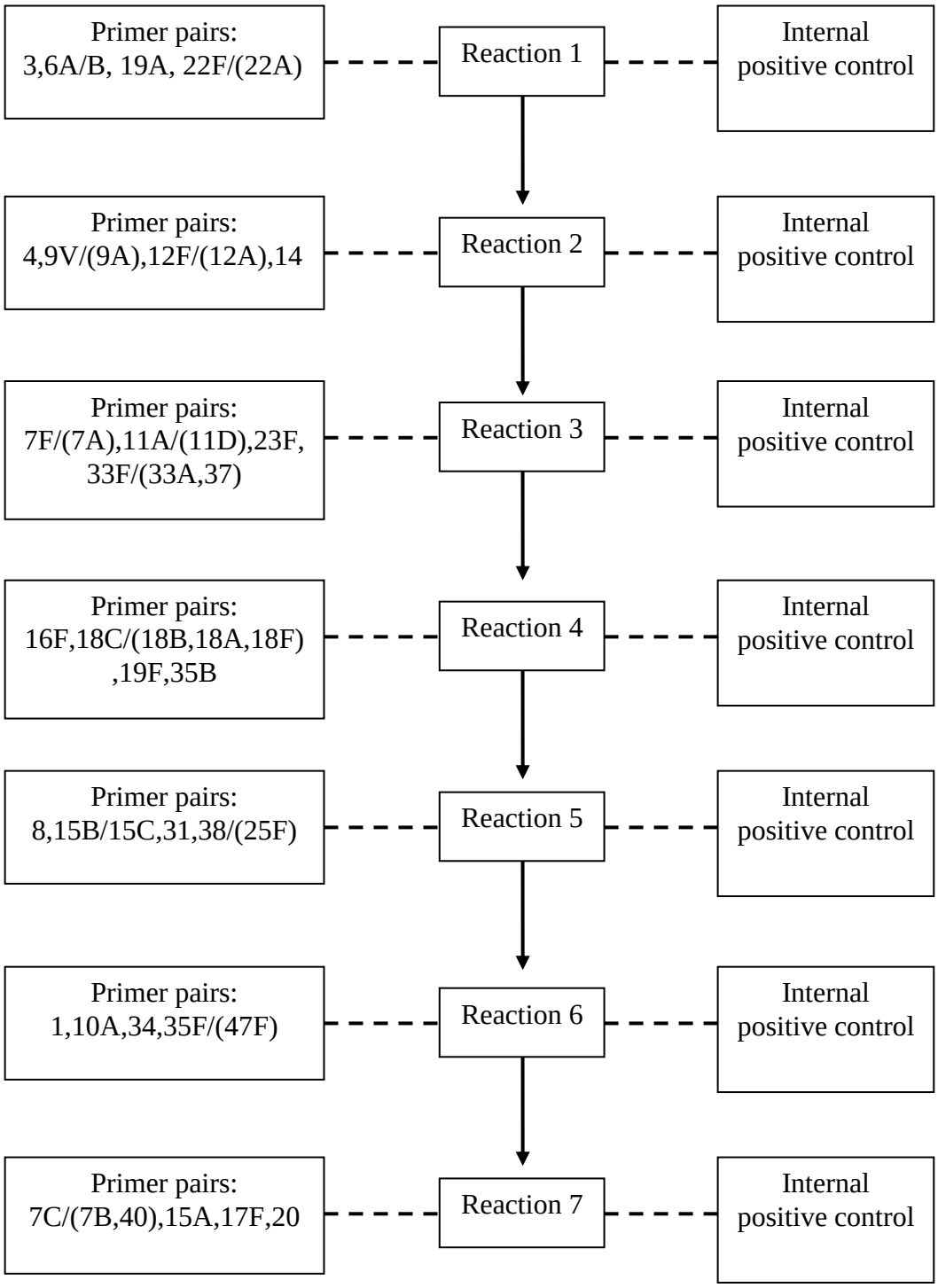


Figure 2.5: The seven multiplex PCR deduction reactions for pneumococcal serotyping. Internal positive control cpsA was simultaneously included in each reaction (Le *et al*., 2011b).

Table 2.1: The seven multiplex PCR deduction pools for pneumococcal serotyping (adapted from Pai *et al.*, 2006)

Reaction	Product size (bp)	Primers	Primer concentration (µM)
1	371	3-f, 3-r	1.5
	250	6A/B-f (biotin), 6A/B-r	0.5
	478	19A-f, 19A-r	1.0
	643	22F-f, 22F-r	1.5
2	430	4-f, 4-r	1.5
	753	9V-f, 9V-r	1.5
	376	12F-f, 12F-r	1.5
	208	14-f, 14-r	1.0
3	826	7F-f, 7F-r	2.0
	463	11A-f, 11A-r	1.0
	384	23F-f, 23F-r	1.5
	338	33F-f, 33F-r	1.0
4	988	16F-f, 16F-r	2.0
	304	19F-f, 19F-r	1.5
	677	35B-f, 35B-r	1.0
	573	sg18-f, sg18-r	1.25
5	294	8-f, 8-r	1.5
	496	15B/C-f, 15B/C-r	1.5
	701	31-f, 31-r	2.0
	574	38-f, 38-r	1.5
6	280	1-f, 1-r	1.5
	628	10A-f, 10-r	1.5
	408	34-f, 34-r	1.5
	517	35F-f, 35F-r	1.5
7	260	7C-f, 7C-r	1.5
	436	15A-f, 15A-r	1.5
	693	17F-f, 17F-r	1.5
	514	20-f, 20-r	1.5

* The cpsA primer targeting the common cps operon region was included in each reaction at 0.5 µM each.

Table 2.2: PCR conditions for multiplex PCR serotyping of *S. pneumoniae*.

Step		Temperature (^{o}C)	Duration
1	Initial denaturation	94.0	4 mins
2	Denaturation	94.0	45 s
3	Annealing	54.0	45 s
4	Extension	65.0	2 mins 30 s
Step (2) to (4) were repeated for a total of 30 cycles.			
5	Final extension	72.0	2 mins

2.4. Peptides design

2.4.1. Selection of lead peptides

A Small Peptides Library was set up by searching into two publicly available databases: RCSB Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do) and Antimicrobial Peptide database (APD) (http://aps.unmc.edu/AP/main.php). Potential AMPs including the naturally occurring or the synthetic variants described in the literature with experimentally determined antibacterial activity were also included. These peptides were termed natural AMPs. No preferred selection on the physicochemical properties of AMPs, however, anionic AMPs were not included and only AMPs with chain length of 30 amino acids or less were of interest due to the better simplicity in designing.

With the initial list of 54 natural AMPs, the next step was to narrow down and select a subset of peptides with the highest likelihood of having inhibitory effects against the target organism *S. pneumoniae*. Since the binding compatibility between these natural AMPs and PBPs have not been previously studied using X-ray crystallization and NMR methods, hence the actual structure of the complex cannot be defined, thus the need for binding prediction. The .pdb structural files of natural AMPs and PBPs were identified and downloaded from PDB. The ZDock server (http://cagt.bu.edu/page/ZDOCK_download) was utilized to predict the potential binding compatibility between the natural AMPs and the six pneumococcal PBPs (Table 2.3). ZDock uses a fast fourier transform method to search for all possible binding modes based on shape complementarity, desolvation energy, and electrostatics. The application shortlisted the top 2000 possible structural combinations (PSCs) protein complexes and generated output data files showing the structural position in three-dimensional space. This application was then executed for all 324 (6 PBPs x 54 natural AMPs) combinations.

In the next stage, we set out to elucidate the predictions using BioMoDroid algorithm (Rathinam, personal communication). The algorithm assumes the fact that the stronger the bond between the natural AMPs and the target, the closer it would be in structural space. The software was used to calculate the distance, d, between the centroid of each natural AMP and its PBPs in each PSC (output from the previous application). The application was written in C++ computer language and executed on Linux shells on a Intel Quad-Core powered server. The centroid was calculated by averaging the xyz coordinates of each atom of each amino acid in the sequence. This distance value was then used to count the number out of the 2000 PSCs and rank each natural AMPs - PBPs combination. The top ten natural AMPs were shortlisted for further investigations: 1D9J, 1Q71, 1M4F, 1HU5, 1MA2, 1HR1, 1T51, 1PG1, 1X7K, and 1VM4.

Table 2.3: The six target pneumococcal PBPs obtained from PDB for peptide design.

PDB ID	Entry Description	Literature
1K25	PBP2x from a highly penicillin-resistant *S. pneumoniae* clinical isolate	Dessen *et al*., 2001
1PMD	penicillin binding protein 2x	Pares *et al*., 1996
1QME	penicillin-binding protein 2x from *S. pneumoniae* implicated in drug resistance	Gordon *et al*., 2000
1RP5	PBP2x from *S. pneumoniae* strain 5259 with reduced susceptibility to beta-lactam antibiotics	Pernot *et al*., 2004
2C5W	crystal structure of penicillin-binding protein 1a reveals a mutational hotspot implicated in beta-lactam resistance in *S. pneumoniae*	Contreras-Martel *et al*., 2006
2JCI	Structural insights into the catalytic mechanism and the role of *S. pneumoniae* PBP1b (Entry 2JCI obsoleted on 26/5/2010, superceded by 2XD5)	Macheboeuf *et al*. 2009

2.4.2. Design of synthetic AMPs

There are many possible strategies to introduce sequence modifications and no single best method that is superior over another. For each of the natural AMPs selected, one designed AMP was generated by single or combination of the following techniques: single amino acid substitution, addition/deletion, truncation, and/or fragment hybridization. Charge and hydrophobic properties of peptides were the principal physicochemical descriptors in designing designed AMPs as the functions to correlate antipneumococcal activity. Moreover, the introduction of specific amino acids in the respective peptide sequences was carefully examined. Finally, this first group designed AMPs which consisted of two major series of peptides were generated with (DAMP1, DAMP2, DAMP4, DAMP8, DAMP10) and without peptide length alterations (DAMP3, DAMP5, DAMP6, DAMP7). All the designed AMPs have unaltered N-terminal and C-terminal fragment as the template peptides except for DAMP6 to evaluate the effects of C-terminal deamidation on antimicrobial activity of the peptides. ,.

2.4.3. Redesigning synthetic antimicrobial peptides with improved antibacterial activity and cell toxicity

From the first phase peptide design, the hydrophobicity and net charge of peptide was determined as the principal descriptors. Towards redesigning better peptides, DAMP6 and DAMP7 were then used as the template for subsequent redesigning to achieve better peptides with high therapeutic potency and low toxicity. The current approach focused on the binding compatibility between two aligned sequences. BioMoDroid (Rathinam, personal communication) was used to predict and generate new peptides, based on the input sequences, with high charge and hydrophobicity compatibility between the residues.

The procedure began by searching the target PBP receptors for potential binding pocket using Metapocket 2.0 server (Huang, 2009) which is available at http://projects.biotec.tu-dresden.de/metapocket/. For each PBP, the run was computed using a 9.0 angstrom radius of probe to get potential binding site and only the top prediction was selected. The sequences predicted were assumed in close conformation and align in linear form according to the sequence of residues. For example, the prediction on PBP 1K25 using the metapocket server had identified the top potential pocket of 32 residues KRKVPTRRMEEIVGNARPIVVGLLIPDMYWTN. The predicted binding pocket for all 6 PBPs ranged from 24 - 32 amino acids in length (Table 2.4). All PBPs used in previous virtual screening served as target receptors, except for 1PMD which contains only CA atoms and is unsuitable to run on the server due to too many grid points produced for the clustering as this file. This receptor file was replaced with another *S. pneumoniae* PBP2X receptor file 2Z2L (Yamada *et al*., 2003).

To predict the binding compatibility between each residual amino acid on the ligand peptide and receptor protein, the hydrophopathy compatibility index (HCI) and charge compatibility index were used to characterize the strength of binding between amino acid residues (Biro, 2006). The HCI was calculated based on the Kyte and Doolittle hydropathy index (the first scale) (Kyte & Doolittle, 1982) and the normalized consensus hydrophobicity scale (second scale) by Eisenberg *et al*. (Eisenberg *et al.*, 1984) using the formula:

$$HCI = 20 - | [HM(A) - HM(B)] \times 19/10.6] |$$

The formula for CCI (third scale):

$$CCI(AB) = 11 - [pI(A)-7] [pI(B)-7] \times 19/33.8$$

The compatibility between amino acid A, and B are characterized by a scale range from 1 (not compatible) and 20 (highly compatible). For each amino acid, all three scales were combined to generate a consensus scale with 25:25:50 score distribution for each scale respectively. The scores were then normalized from 0 (non-compatible) to 1 (highly compatible).

Both the binding pocket sequence of the receptor and template peptide was aligned in parallel from N-terminal to C-terminal. Pairing involving the 1st N-terminal residue of the pocket with the first N-terminal residue of peptide designated as position 1/first iteration. For the 2nd position/iteration, the peptide was shifted one residue toward the C-terminal and the pairing involves the 2nd N-terminal residue of the pocket with the first N-terminal residue of peptide. The iterations will then be continued for the remaining residues. Thus,

$$\text{Total no. of positions/iteration} = \text{total no. of pocket residues} - \text{total no. of peptide residues} + 1$$

The binding algorithm is based on a novel method call Double Y. In this method, the primary pair (residues reside directly on the opposite between the pocket and peptide) and the secondary pair (residues reside adjacent to the primary pair residue) were summed up to calculate a score. The scores for redundant pairs were ignored. For example, for the pairing between ARK and GLHAPPL, there are three primary pairs (AG, RL, KH) and five secondary pairs (AL, RG, RH, KL, KA) thus giving a total of eight pairs. At the 2nd position/iteration, there are a total of nine pairing with pairs of AG, AL, AH, RL, RH, RA, KH, KA, KP.

The calculations were performed in two continuous cycles. At each cycle, the residual substitutions were framed at a window size of two amino acids. Since there is a

total of 20 amino acids, a total of 400 possible substitutions (and thus the new sequences) are expected from each cycle of calculations for the respective peptide-pocket pairs. Two windows each with a window size of two was framed to allow substantial alteration to the peptide sequence while limiting the changes to a maximum of four residues, which is approximately 30.8% of the template. Higher window size was not attempted as this may result in dramatic changes to peptide sequence. The length of the peptide was preserved at its original length of 13 amino acids as modification without length alteration was shown to be the better designing strategy.

After calculations on all pairs at the first iteration were completed, the peptide was shifted one position towards the C-terminal and a similar calculation was performed until the last iteration at the C-terminal. All scores computed using the normalized scale based on double Y method was tabulated and sorted. The score for each iteration was compared and sequences with the top score was extracted and serve as the template peptide for the second cycle of computation performed using similar procedures on each pocket sequence. Subsequently, the scores for the peptides generated after two cycles of calculations were sorted among other peptides calculated against other pockets. The top three scoring sequences for each DAMP6 and DAMP7 were selected for *in vitro* testing. Peptides generated based on DAMP6 were designated DP61, DP62, and DP63 (DP6 series) and peptides generated based on DAMP7 were designated DP71, DP72, and DP73 (DP7 series). This redesigning also includes 1T51, the only natural AMP found to be highly active against *S. pneumoniae* based on the MIC result and were designated DP51, DP52, and DP53 (DP5 series).

Besides that, a separate series of five synthetic hybrid peptides (DM1, DM2, DM3, DM4, and DM5) were generated by hybridizing fragments of varying length from C-terminus of DAMP6 and N-terminus of DAMP7. However, four of the residues at each terminal were unchanged. This is because DAMP6 was initially designed based on

1VM4 which belongs to the GLFD family of antimicrobial peptide, the four residues was reported to be crucial for its activity (Wang *et al.*, 2005). Also, the four residues at the Arg-rich C-terminal fragment of DAMP7 was responsible for its activity (Selsted *et al.*, 1992). Hence, these fragments were preserved while the central fragments were sequentially substituted. Since both templates were 13 amino acids in length hence the hybrid peptides were similarly restricted to the same length. All designed AMPs generated from the redesigning approach were collectively referred to as second group designed AMPs.

Table 2.4: Pocket site sequences predicted using Metapocket 2.0 server.

Target PBPs	Pocket sequence predicted	Length
1K25	KRKVPTRRMEEIVGNARPIVVGLLIPDMYWTN	32
2Z2L[a]	GSKDWDEHSSNQTKSGTAQYIFSY	24
1QME	NRKVRRMVGNPIVVGTLIEVPDMYWLAN	28
1RP5	NKVPTRRMEEVGPGARPIVVGTIPDW	26
2C5W	WGSTKYNWDQSRNITKTGTSNFVADEL	27
2XD5[b]	ASKMYANYSWNMTKTGTTNQDNMQY	25

[a]1PMD which contains only CA atoms in the .pdb structural file could not be executed on the Metapocket 2.0 server due to the excessive amount of grid points generated for the clustering of this file. Replaced by 2Z2L (Yamada *et al.*, 2007).
[b]superceded 2JCI.

2.4.4. Peptides physicochemical analytical tools

Five molecular parameters describing the charge and hydrophobicity nature of peptides were used to characterize the physicochemical properties of both natural AMPs and designed AMPs: total net charge (NetC), Charge density (ChD), hydrophobic ratio (HR), total hydrophobic value (THV), and grand average of hydropathy (GRAVY). The ExPASy ProtParam tools (http://web.expasy.org/protparam/) and APD were used to perform the physicochemical analysis for each peptide. NetC was calculated by subtracting the sum of positively-charged amino acids (Arg, Lys, His) by the sum of negatively-charged amino acids (Asp, Glu). C-terminal amidation was assigned with one positive charge (+1). ChD was calculated by dividing NetC with the chain length of peptide. HR is the percentage of hydrophobic amino acid (Ile, Val, Leu, Phe, Cys, Met, Ala, Trp) in the peptide chain. THV was computed by summation of hydrophobicity value for each residue based on the Kyte and Doolittle hydropathy index (Kyte & Doolittle, 1982). GRAVY was the mean hydrophobicity value of the peptide, calculated by dividing THV by the number of residues.

2.4.5. Peptide synthesis and testing

All natural AMPs and the first group of designed AMPs were synthesized by Sigma Co. (Japan) except 1VM4 by Genscript (USA). The second group of designed AMPs was synthesized by Genscript (USA). Peptide synthesis was performed using 9-fluorenylmethoxycarbonyl for solid phase peptide synthesis. Quality analyses of peptides were validated using high performance liquid chromatography and mass spectrometry. All peptides were then tested for *in vitro* antimicrobial and cell toxicity. Peptides for use in *in vitro* testing were synthesized to > 90% purity; peptides for use in *in vivo* therapeutic efficacy and toxicity studies in mice were synthesized to > 95% purity.

2.5. *In vitro* assessment of peptides

2.5.1. Bacterial cultures and assay medium

Twenty *S. pneumoniae* clinical isolates from each penicillin-susceptibility group (resistant, PRSP; intermediate, PISP; susceptible, PSSP) were obtained from a retrospective study (Palanisamy, 2008) which represents a subset of isolates used in the serotyping. Sheep blood agar (Oxoid, UK) was used for pneumococcal cultivation purpose. Nutrient agar (NA) (Oxoid, UK) was used for *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *P. aerugenosa* ATCC 15442, *Acinetobacter baumanii* ATCC 15308, and one clinical isolate for each methicillin-resistant *S. aureus* (MRSA), *Enterococcus cloacae, Citrobacter* spp., and *K. pneumoniae*. For antimicrobial testing, MHA and cationically-adjusted MHB (CAMHB) were used. All agar medium for *S. pneumoniae* was supplemented with 5% (v/v) defibrinated sheep blood (Oxoid, UK). The bacterial cultures were stored in multiple vials in Brain Heart Infusion Broth (BHIB) (Oxoid, UK) supplemented with 10% (v/v) glycerol at -80^{o}C to avoid repeated freeze-thawed cycles on the cells. All freeze-stocked strains were passaged twice prior to experimentation.

2.5.2. Broth microdilution assay

Procedures were performed to determine the minimum inhibitory concentration (MIC) of peptides according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2008) to determine the MIC of peptides. Bacterial strains were grown for 18 - 24 hrs at 37^{o}C under 5% CO_2. Direct suspension of the colonies were made in CAMHB and adjusted to OD_{625} 0.08 - 0.1 which corresponds to 1 ~ 2 x10^8 colony forming units (CFU)/ml followed by serial ten-fold dilutions to give 1x10^6 CFU/ml supplemented with 10% (v/v) laked horse blood (LHB) (Oxoid, UK). Fifty microliters of bacterial suspension was then aliquoted to 96-well microtiter plates (Corning, USA) containing equal volume of serially diluted peptides to give final concentrations of peptides

encompassing the range of 1.96 – 250 µg/ml. The MIC value for inactive peptides producing no inhibition in the range tested was denoted as > 250 µg/ml. The plates were incubated for 18 - 24 hrs at 37^{o}C under 5% CO_2. MIC was read as the concentration of peptide producing complete inhibition on the visible growth of the test organism. Results were pooled from three independent experiments with no more than one two-fold dilution in variation. MIC value that appears at least twice was taken as the final MIC for the particular isolate/peptide. Effective range was defined as the range of concentrations where the peptides produce detectable activity. Effective percentage was the proportion of organism inhibited within the effective range. Since the isolates were obtained prior to year 2008 where major revision in antibiotic susceptibility breakpoints were introduced, the previous (prior 2008) penicillin susceptibility breakpoint was followed which corresponds to the penicillin (oral penicillin V) breakpoint of the revised criteria.

The broad spectrum antibacterial activity of the peptides against a panel of eight clinically important bacteria were also tested, this includes *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 15442, *Acinetobacter baumanii* ATCC 15308, and one clinical isolate for each methicillin-resistant *S. aureus* (MRSA) *Enterococcus cloacae, Citrobacter* spp., and *K. pneumoniae*.

2.5.3. Bacterial killing assay

This assay was performed to evaluate the killing kinetics of peptides (Eckert *et al.*, 2006). One PRSP, PISP, and PSSP isolate were each grown and prepared to 5 x 10^6 CFU/ml. Each isolate in CAMHB was challenged with peptides at two times the respective MIC values. At the indicated time intervals (0, 30, 60, 120, 150, 180, 210, and 240 mins; “0” indicate pretreatment), 10 µl of suspension was removed and immediately serially diluted in PBS on ice to block further antibacterial activity and

bacterial growth and spread-plated on MHA to obtain viable colony counts. The percentage (%) surviving cell was calculated by dividing the surviving CFU of the treated cells over the surviving CFU of the untreated control cells at the respective time points. Results were pooled from three independent experiments and expressed as mean ± standard deviation (SD). Penicillin was also included to evaluate the killing kinetics of peptides in comparison to this commonly use conventional antibiotic against *S. pneumoniae*.

2.5.4. Synergism study

Synergism assay was performed using the checkerboard method (Bajaksouzian *et al.*, 1996) with minor modification. In order to prepare a range of concentrations which allows simultaneous detection of antagonism, indifference/additive, and synergism, the assay was performed in such a way that each column contained a fixed 0.25X MIC of the first peptide and 12 serial two-fold dilutions of the second peptide at each row beginning at 8X MIC. This yielded 12 peptide-peptide (peptide A: peptide B) combinations at varying ratios from 1 : 128 to 16 : 1. The combination effect of peptides was defined according to the fractional inhibitory concentration (FIC) index, whereby

$$\text{FIC} = \frac{\text{MIC of peptide A in combination}}{\text{MIC of peptide A alone}} + \frac{\text{MIC of peptide A in combination}}{\text{MIC of peptide A alone}}$$

Two peptides were antagonistic if FIC > 4.0, indifference if $0.5 < \text{FIC} \leq 4.0$, and synergism if ≤ 0.5 (Bajaksouzian *et al.*, 1996). Only peptides with antipneumococcal MICs within the range of concentrations tested were selected. This included DAMP6, DAMP7, DP71, DP72, DM1, DM2, DM3, DM4, and DM5. Penicillin was also included

to investigate the possible synergism the designed AMPs have with conventional antibiotic. One isolate from each PRSP, PISP, and PISP were selected and inoculums were prepared according to the broth microdilution assay without the addition of LHB to allow better visible observation of well clearance.

2.5.5. Transmission electron microscopy

An overnight culture of selected PRSP on sheep blood agar was passaged twice and directly suspended in CAMHB. As sample for Transmission Electron Microscopy (TEM) required high cell density of about 10^8 to 10^{10} to be viewable (Hartmann *et al.*, 2010), the bacteria was prepared to 5 x 10^{10} CFU/ml and treated with supra-concentration of peptides at 8 mg/ml for four hours at 37^{o}C under 5% CO_2. Cells treated with only water was served as the untreated control. For TEM sample preparation, standard protocol provided by the Electron Microscopic Unit, Faculty of Medicine, University of Malaya was followed. The cells were washed twice with CAMHB before overnight fixation in 4% (v/v) glutaraldehyde, two times postfix washes with cacodylate buffer, incubate, two hours incubation with osmium tetroxide buffer (OsO_4 1: 1 cacodylate), and washed twice with cacodylate buffer before overnight incubation in the same buffer. Next, the samples were washed twice with double distilled water, 10 mins of uranyl acetate incubation, and washed twice with double distilled water before subjected to dehydration by gradual ethanol series: 35% for 10 mins, 50% for 10 mins, 70% for 10 mins, 95% for 15 mins, and three rounds of absolute (100%) ethanol for 15 mins. Following these, samples were incubated with two rounds of propylene oxide for 15 mins, propylene oxide 1 : 1 Epon for 1 hr, propylene oxide 1 : 3 Epon for 2 hrs, overnight incubation with Epon, embedded in Agar 100 resin at 37^{o}C for five hours, and maintained in 60^{o}C until viewing. Ultrathin sectioning were prepared on Reichert Ultramicrotome, copper grids 3.05 mm (300 square mesh) (agar Scientific), and stained

with ethanol-based uranyl acetate and lead citrate for 5 mins. TEM viewing was performed with Leo Libra 120 under standard operating conditions.

2.5.6. Hemolytic activity

Hemolytic assay was performed as previously described (Zelezetsky *et al.*, 2005). Freshly drawn human erythrocytes were rinsed three times with PBS and resuspended in PBS to 4% (v/v). One hundred microliters of the suspension was added to 96-well microtiter plate (Corning, USA) containing equal volume of peptides to give final concentrations of peptides encompassing the range of 1.96 – 250 µg/ml. PBS and 0.1% (v/v) Triton-X 100 were used as 0% and 100% hemolytic control respectively. Plates were incubated at 37^{o}C for 1 hr. Subsequently, the plate was centrifuged and the supernatant was transferred to a new plate. The release of hemoglobin in the supernatant was monitored at absorbance of 450 nm using Glomax Multidetection system (Promega, USA). Results were pooled from three independent experiments and expressed as mean ± SD. HC_{10} and HC_{50} were defined as the peptide concentrations causing 10% and 50% hemolysis on human erythrocytes, respectively. H_{max} was the percentage hemolysis observed at the maximum concentration as defined throughout this study (all peptides 250 µg/ml, PEN 4 µg/ml).

2.5.7. Cytotoxicity against human cell lines

Both the NL20 human lung bronchial normal epithelial cell line and the A549 human lung alveolar adenocarcinoma epithelial cell line were used for cell cytotoxicity tests. Besides investigating the cytotoxicity of peptides, differences in cell viability between the two cell lines can be used to evaluate the cell line selectivity and to assess potential anticancer potential of the peptides. NL20 cell line was grown in Ham's F12 medium (Lonza, USA) and A549 cell line was grown in Roswell Park Memorial

Institute medium (RPMI-1640, Hyclone, USA). Both media were supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Australia) as growth medium for cell culture purposes or with 2% (v/v) FBS as maintenance medium in cell cytotoxicity testing. The assay was performed as described by Lee *et al*. (Lee *et al.*, 2011b). NL20 cells was seeded overnight at 3 x 10^4 cells/well in 96-well cell culture-treated flat bottom microtiter plate (Corning, USA) and treated with serial dilutions of peptide at a final concentration of 1.95 - 250 μg/ml. Penicillin was tested from 0.03 – 4 μg/ml, the same range used to classify the susceptibility of pneumococcal isolates in this study. Test using A549 cells followed the same procedures with 1 x 10^4 cells/well. Plates were incubated at 37^{o}C under 5% CO_2 for 24, 48, and 72 hrs. Cell viability was detected by using CellTiter 96® AQueous Non-Radioactive Cell Proliferation assay (Promega, USA) and the colorimetric changes were read with Glomax multidetection system (Promega, USA). CellTiter 96® AQueousNon-Radioactive Cell Proliferation assay is composed of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine methosulfate; PMS). The dehydrogenase enzymes in metabolically active cells will convert the MTS into soluble formazan product which produces calorimetric change that can be quantitated directly by absorbance measurement at 490 nm. Results were pooled from three independent experiments and expressed as mean ± SD. IC_{50} was defined as the peptide concentration which resulted in 50% cell viability. I_{max} was the percentage of cell viable treated with peptides/penicillin at the maximum concentration as defined throughout this study (all peptides 250 μg/ml, penicillin 4 μg/ml).

2.6. *In vivo* assessment of peptides

2.6.1. Animal maintenance and handling

The mouse represents the choice of animal model to study the therapeutic efficacy of novel drugs as they are the most susceptible animals to pneumococcal infections (Amory-Rivier *et al.*, 1999) and are easy to handle as compared to other animals. Male ICR mice (4 weeks old) were used throughout the *in vivo* studies. The mice were acclimatized for seven days prior to any experimentation and allowed mouse feeding pellet and water *ad libitum*. Procedures involving subcutaneous (SC) and intranasal (IN) routes which requires anesthesia were given the standard dose of ketamine (Narketan®-10, 100 mg/kg) and xylazine (ilium xylazil-20, 10 mg/kg) combination via intraperitoneal (IP) injection. All animal handling and experimental procedures were approved by the Faculty of Medicine Animal Care and Use Committee of University of Malaya, ethic number MP/05/05/2010/LCF(R).

2.6.2. Injection/administration procedures

All injections were performed using Myjector 1 ml U100 29G insulin syringe (Terumo). The mouse was held head-down and IP injection be given to the lateral side at lower-left quadrant of the abdomen. The posture was maintained for 15s before returning to the normal position. SC injection was given to the left thigh and followed by right thigh if repeated injections were required. Intrathoracic (IT) injection was given to the lateral side of the right thorax through the intercostals muscles between the adjacent ribs. Procedures for IN route used a long-tailed blunt-end 10 µl tips attached to a 1 ml syringe (Terumo) and be given through either nostril with head-up position. This position was maintained for 30 s with occasional assisted-closing of the mouth to facilitate inhalation.

2.6.3. *In vivo* toxicity assessment

In vivo toxicity was performed to determine the possible side effects associated with the administration of peptides in mice. The five hybrid DMs were selected for *in vivo* testing due to their promising *in vitro* antipneumococcal and toxicity profiles. The mice were randomized and three to four mice per group were administered with the respective peptides at 2 hrs, 12 hrs, and 24 hrs for three dosing (three dose regimen) via SC, IN, and IP routes. The peptides were initially given at high dose (100 mg/kg via IP and SC routes, 20 mg/kg via IN route). Any physical or behavior abnormality was recorded and survival of mice was noted. In the events where adverse effects including highly physical stresses, highly lethargic, highly physical inactiveness, and/or death were observed in the mice, lower graded step-down doses were given. Dose administered via IN route was limited by the low volume (20 µl) deliverable through the nasal cavity of mouse and thus the highest dose given was 20 mg/kg. Untreated control group was administered with sterile distilled water only. The mice were observed for seven days or until dead/moribund, whichever earlier. At the seventh day, all mice were sacrificed and blood was collected via cardiac puncture using 25G syringe (Terumo) attached to 1 ml needle (Terumo). Whole blood for complete haematogram was collected in 500 µl dipotassium EDTA microtainer tube (BD bioscience, USA). Blood collected in another 1.5 ml tube was centrifuged at 8,000 rpm for 5 mins and the serum was aliquoted to a fresh 1.5 ml tube for serum biochemistry analysis. The whole blood and serum samples were transported on ice and sent to the haematology and clinical biochemistry laboratory, Veterinary Laboratory Service Unit (VLSU), Faculty of Veterinary Medicine, Universiti Putra Malaysia for analysis. The whole blood parameters analyzed were number of red blood cells (RBC), white blood cells (WBC), B neutrophil, S neutrophil, lymphocytes, monocytes, eosinophil, basophil, and thrombocytes, hemoglobin (Hb) concentration, packed cell volume (pCV), mean

corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), and plasma protein concentration. Serum biochemistry analysis included the concentrations of alanine transaminase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), creatinine, urea, lactate dehydrogenase (LDH), and direct and total bilirubin. Major organs (brain, lung, liver, kidney, spleen) were harvested and fixed in 10% (v/v) buffered formalin for at least seven days. Histological tissues processing with hematoxylin and eosin stain were processed at the histopathology laboratory, VLSU, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

2.6.4. *In vivo* therapeutic efficacy assessment

2.6.4.1. Development of lethal murine pneumococcal infection models

Each selected peptide was to be tested in two pneumococcal infection models; the pneumococcal systemic infection model which mimicked pneumococcal bacteremia in human and the pneumococcal pneumonia model which mimicked the pneumococcal pneumonia in human. Towards developing the pneumococcal infection models, the specific strain used and the inoculums must be optimized carefully prior to therapeutic testing. This is because the *in vivo* virulence (disease-causing ability and time to death) of *S. pneumoniae* varied considerably among different strains, routes of infection, and the inoculums used (Amory-Rivier *et al.*, 1999; Benton *et al.*, 1997; Briles *et al.*, 1992).

Three PRSP strains were first assessed for its virulence to cause lethal infections in mice (n = 3). The selected PRSP strains were subcultured twice on sheep blood agar at 37^{o}C under 5% CO_2 for 18 – 24 hrs and adjusted to OD_{625} 0.08-0.1 (approximately 1 ~ 2 x 10^8 CFU/ml) in BHIB. The suspension was then serially diluted to appropriate inoculums on ice and immediately be inoculated into the mice at the desired routes. To induce systemic infection in mice, 100 µl of the inoculums was given at 1.5 x 10^4, 1.5 x 10^3, 1.5 x 10^2, and 1.5x10^1 CFU/mouse via IP route. To induce pneumonia in mice, 50

µl of the inoculums were given at $5x10^4$, $5x10^3$, $5x10^2$, and $5x10^1$ CFU/mouse via IT route. The IT infection was able to induce consistent and reproducible lethal infection. Following inoculations, the number of cells inoculated in the mice was confirmed by plate count on sheep blood agar. Although inducing pneumonia infection in mice via the IN route of infection was attempted, however, none of the strains or inoculums to as high as $5x10^7$ CFU/mouse was able to induce infection. Uninfected control group (medium only) with equal number of mice as the test groups were included in each trial. From the optimization, one particular strain (SP12) was selected. The inoculum used to induce lethal systemic infection was 1.5×10^2 CFU/mouse which cause 100% mortality between day 2 to day 4 postinfection. The inoculum used to induce lethal pneumonia infection was 5×10^3 CFU/mouse which caused 100% mortality between day 2 to day 4 postinfection. Two other strains were either avirulent in inducing lethal infections or were unable to produce 100% mortality within the time frame.

2.6.4.2. Determination of survival rates in infected mice following treatment with peptides

All mice were randomized before receiving the first treatment dose and be divided into the respective groups. The selected dose and regimens of treatment of the respective peptides were first tested using groups of three mice to determine potential peptides with protective effects in the two infection models. Selected peptides were then tested in a larger group (n = 10) for detailed evaluation. Graded doses of penicillin were also tested to determine the therapeutic efficacy of this standard antibiotic. Uninfected control (medium only) and untreated control (inoculums only) with equal number of mice as the test groups were included in each trial and treated with sterile distilled water only.

2.6.5. *In vivo* therapeutic synergism with penicillin

After determining the *in vivo* therapeutic efficacy of peptides in standalone form, the protective effect of candidate peptides in combination with the standard antibiotic penicillin was carried out. The therapeutic efficacy of penicillin against the mouse infection model was determined for doses of 10 mg/kg. 20 mg/kg, 40 mg/kg, and 80 mg/kg following the same procedures and treatment regimen as the DMs. To allow the detection of therapeutic synergism, doses of penicillin and peptide which produced minimal survivability in the infected mice were chosen and prepared to 2X the desired testing concentration at 0.1 ml in separating tubes. Immediately before treatment, both drugs were mixed together and this would dilute both the drugs to the desired testing doses at final volume of 0.2 ml. The therapeutic efficacy was then performed in the infection models (n = 10).

2.7. Statistical analysis

Statistical analysis was performed using SPSS 16. Association among serotypes, clinical sites of isolates, age groups, and penicillin susceptibility were tested using Chi-square test of independence or Fisher's exact test, whenever appropriate. When these tests yielded significant association ($p \leq 0.05$), adjusted residual value of $\geq +2$ or ≤ -2 was used to determine the underlying positively or negatively associated groups, respectively. For *in vivo* toxicity testing, the statistical difference in whole blood haematogram and serum biochemistry parameters between the treated versus the untreated control groups were conducted using One-way ANOVA with *post-hoc* Dunnett-t test. For *in vivo* therapeutic efficacy and synergism testing, survival analysis of each treated versus untreated control groups were performed using Kaplan-Meier analysis with log-rank test (Mantel-Cox). Survival plots were generated from the SPSS based on Kaplan-Meier analysis.

CHAPTER 3

RESULTS

3.1. Antibiotic susceptibility and multiplex serotyping of *S. pneumoniae*

Prior to year 2008, the penicillin susceptibility breakpoint for *S. pneumoniae* was collectively defined by penicillin-susceptible *S. pneumoniae* (PSSP, MIC ≤ 0.06 μg/ml), penicillin-intermediate *S. pneumoniae* (PISP, MIC = 0.12 – 1 μg/ml), and penicillin-resistant *S. pneumoniae* (PRSP, MIC ≥ 2 μg/ml) (Clinical and Laboratory Standards Institute, 2005). From year 2008 onwards, the penicillin susceptibility breakpoint has undergone major revision. The penicillin interpretive criteria for *S. pneumoniae* are now classified into three subgroups; penicillin (oral) group follows the same penicillin susceptibility classification as the pre-revision breakpoint: PSSP (MIC ≤ 0.06 μg/ml), PISP (MIC = 0.12 – 1 μg/ml), and PRSP (MIC ≥ 2 μg/ml); parenteral penicillin for meningitis isolates defines *S. pneumoniae* as PSSP (MIC ≤ 0.06 μg/ml) and PRSP (MIC ≥ 0.12 μg/ml) only and has no PISP grouping; major change is the parenteral penicillin for nonmeningitis isolates which defines PSSP (MIC ≤ 2 μg/ml), PISP (MIC = 4 μg/ml), and PRSP (MIC ≥ 8 μg/ml) following higher MIC values for the respective susceptibility groups (Table 3.1). This also means that the previously defined PSSP, PISP, and PRSP (MIC = 2 μg/ml) will now be classified as PSSP according to the revised breakpoint.

In this study, since the pneumococcal isolates were collected prior to year 2008 (1999 – 2007), the isolates were classified using the pre-revision breakpoint (Clinical and Laboratory Standards Institute, 2005), which is also equivalent to the revised penicillin (oral) breakpoint. Of the 151 isolates, there were 21.2% (32/151) PRSP, 29.1% (44/151) PISP, and 49.7% (75/151) PSSP (Table 3.2, highlighted in yellow). The ratio of penicillin susceptible to nonsusceptible isolates (PNSP) was at near equal (PSSP 49.7%, PNSP 50.3%). In addition, 42.6% (20/47) of isolates obtained from patients below 5 years old were PSSP and 27.7% (13/47) were PRSP (Table 3.3, highlighted in yellow). On the other hand, a large proportion (60.6%, 20/33) of the isolates from

elderly adults aged ≥ 60 were PSSP and only 15.2% (5/33) were PRSP (Table 3.3, highlighted in blue). Additionally, a great majority of PISP (79.5%, 35/44) and PRSP (81.2%, 26/32) were isolated from noninvasive sites although no statistical significant association was noted ($p = 0.100$). Altogether, they constituted 80.3% (61/76) of the PNSP isolates. No significant association was observed between penicillin susceptibility and age of patients ($p = 0.524$) as well as invasiveness of isolates ($p = 0.07$). Nonetheless, clinical sites of pneumococcal isolations were highly associated ($p < 0.001$) with age of patients (Table 3.4). Most (80.9%, 38/47) of the isolates from children < 5 years old were noninvasive among which 73.7% (28/38) of these isolates were sourced from the NP site (59.6%, 28/47) (Table 3.4, highlighted in yellow). In contrast, a significant proportion (42.4%, 14/33) of isolates from the elderly adult ≥ 60 years old was obtained from blood (Table 3.4, highlighted in green).

A representative complete seven multiplex reactions performed on 10 PRSP isolates was shown in Figure 3.1. Serotypes detected among the Malaysian isolates were 1, 3, 10A, 11A/11D, 12F/12A, 14, 15A, 15B/15C, 16F, sg18(18C/18B/18A/18F), 19A, 19F, 23F, 35B, 35F/47F, 6A/6B, 7C/7B/40, 7F/7A, 9V/9A, 34 (Table 3.2). Serotype 19F was the most prevalent serotype (37.1%, 56/151) followed by serotype 23F (10.6%, 16/151), serotype 1 (6.0%, 9/151) and serotype 6A/6B (6.0%, 9/151) (Table 3.2, highlighted in green). Together, serotype 19F and 23F accounted for almost half of all cases (47.7%, 72/151). The majority of serotype 19F (78.6%, 44/56) were nonsusceptible to penicillin and approximately half of the serotype 19F PNSP were PRSP (45.5%, 20/44). Statistical analysis showed significant relationship between serotype and penicillin susceptibility ($p < 0.001$). In particular, serotype 19F was significantly associated with PNSP while serotype 19A was associated with PSSP (Table 3.2, highlighted in blue). Besides this, a pool of less prevalent serotypes was detected including serotypes 3, 10A, 12F/12A, 15A, 15B/15C, 16F,

sg18(18C/18B/18A/18F), 7C/7B/40, 7F/7A while the nontypeable pneumococci constituted 13.2% (20/151) of the total isolates collected in the current study.

A large proportion (72.2%, 109/151) of the clinical isolates was obtained from noninvasive sites (Table 3.4). Among the noninvasive sites, sputum (27.8%, 42/109) and NP (25.2%, 38/109) constituted the two main sites of pneumococcal isolations (Table 3.4, highlighted in purple). Together with *S. pneumoniae* from blood cultures (Table 3.4, highlighted in blue), these three sites represented the major sources of pneumococcal isolations which accounted for 77.5% (117/151) of all isolates. Other clinical sites include tracheal secretion (7.9%), ear swab/pus (6.0%), pleural fluid (2.0%), eye swab/pus (2.0%), cerebrospinal fluid (1.3%), broncheoalveolar lavage (1.3%), and others (2.0%). Isolates from rare sites such as Bartholin's abscess and vaginal discharge were reported in this study as well. Pneumococcal serotypes were found to be highly associated ($p = 0.001$) with invasiveness of the isolates (Table 3.5). Among them, serotype 19A was associated with invasive sites (75%, 6/8) while serotypes 19F was determined to be the dominant serotype (89.3, 50/56) in noninvasive sites (Table 3.5, highlighted in yellow).

On the other hand, statistical testing showed significant relationship ($p = 0.03$) between the age of patients and serotypes (Table 3.6). Patients aged 5 – 59 years old represented the largest age group which accounted for approximately half of all cases (47.0%, 71/151). A large proportion of serotype 19A (75.0%, 6/8), 23F (75.0%, 12/16) and 6A/6B (66.7%, 6/9) were isolated from patients within this age group (Table 3.6, highlighted in yellow). Serotype 19F constituted the major serotype (46.8, 22/47) among children < 5 years old (Table 3.6, highlighted in blue).

Table 3.1: *S. pneumoniae* penicillin susceptibility breakpoints before and after year 2008 revisions.

	MIC (μg/ml)						
	Before revision			After revision			
	S	I	R		S	I	R
Penicillin	≤ 0.06	0.12 - 1	≥ 2	Penicillin parenteral (nonmeningitis)	≤ 2	4	≥ 8
				Penicillin parenteral (meningitis)	≤ 0.06	-	≥ 0.12
				Penicillin (oral penicillin V)	≤ 0.06	0.12 - 1	≥ 2

Table 3.2: Distribution of pneumococcal serotypes with respect to penicillin susceptibility.

Serotype[a]	Frequency (adjusted residual, % within serotype, % within penicillin susceptibility group)												Total	
	Susceptible				Intermediate				Resistant					
1	7	(1.7,	77.8,	9.3)	2	(-0.5,	22.2,	4.5)	0	(-1.6,	0.0,	0.0)	9	(6.0)
3	1	(1.0,	100.0,	1.3)	0	(0.6,	0.0,	0.0)	0	(-0.5,	0.0,	0.0)	1	(0.7)
14	1	(-1.3,	20.0,	1.3)	4	(2.5,	80.0,	9.1)	0	(-1.2,	0.0,	0.0)	5	(3.3)
34	0	(-1.4,	0.0,	0.0)	0	(-0.9,	0.0,	0.0)	2	(2.7,	100.0,	6.3)	2	(1.3)
10A	1	(1.0,	100.0,	1.3)	0	(-0.6,	0.0,	0.0)	0	(-0.5,	0.0,	0.0)	1	(0.7)
11A/11D	1	(-0.6,	33.3,	1.3)	2	(1.4,	66.7,	4.5)	0	(-0.9,	0.0,	0.0)	3	(2.0)
12F/12A	3	(1.8,	100.0,	4.0)	0	(-1.1,	0.0,	0.0)	0	(-0.9,	0.0,	0.0)	3	(2.0)
15A	1	(1.0,	100.0,	1.3)	0	(-0.6,	0.0,	0.0)	0	(-0.5,	0.0,	0.0)	1	(0.7)
15B/15C	1	(1.0,	100.0,	1.3)	0	(-0.6,	0.0,	0.0)	0	(-0.5,	0.0,	0.0)	1	(0.7)
16F	2	(1.4,	100.0,	2.7)	0	(-0.9,	0.0,	0.0)	0	(-0.7,	0.0,	0.0)	2	(1.3)
sg18	2	(1.4,	100.0,	2.7)	0	(-0.9,	0.0,	0.0)	0	(-0.7,	0.0,	0.0)	2	(1.3)
19A	7	(2.2,	87.5,	9.3)	1	(-1.1,	12.5,	2.3)	0	(-1.5,	0.0,	0.0)	8	(5.3)
19F	12	(-5.3,	21.4,	16.0)	24	(2.8,	42.9,	54.5)	20	(3.4,	35.7,	62.5)	56	(37.1)
23F	9	(0.6,	56.3,	12.0)	1	(-2.1,	6.3,	6.3)	6	(1.7,	37.5,	18.8)	16	(10.6)
35B	2	(0.0,	50.0,	2.7)	2	(0.9,	50.0,	4.5)	0	(-1.1,	0.0,	0.0)	4	(2.6)
35F/47F	2	(0.6,	66.7,	2.7)	1	(0.2,	33.3,	2.3)	0	(-0.9,	0.0,	0.0)	3	(2.0)
6A/6B	3	(-1.0,	33.3,	4.0)	5	(1.8,	55.6,	11.4)	1	(-0.8,	11.1,	3.1)	9	(6.0)
7C/7B/40	1	(1.0,	100.0,	1.3)	0	(-0.6,	0.0,	0.0)	0	(-0.5,	0.0,	0.0)	1	(0.7)
7F/7A	3	(1.8,	100.0,	4.0)	0	(-1.1,	0.0,	0.0)	0	(-0.9,	0.0,	0.0)	3	(2.0)
9V/9A	0	(-1.0,	0.0,	0.0)	0	(-0.6,	0.0,	0.0)	1	(1.9,	100.0,	3.1)	1	(0.7)
NT	16	(2.9,	80.0,	21.3)	2	(-2.0,	10.0,	4.5)	2	(-1.3,	10.0	6.3)	20	(13.2)
Total	75	(49.7)			44	(29.1)			32	(21.2)			151	

[a]Fisher's exact test found significant association ($p < 0.001$) between serotypes and penicillin susceptibility of pneumococcal isolates (significant at $p \leq 0.05$). Adjusted residual value of $\geq +2$ or ≤ -2 was used to determine the underlying positively or negatively associated groups, respectively.
Highlighted in yellow: The number (percentage) of penicillin susceptible, intermediate, and resistant pneumococcal isolates.
Highlighted in green: The four major serotypes detected in the order of 19F, 23F, 1 and 6A/B.
Highlighted in blue: Serotypes positively or negatively associated with penicillin susceptibility of pneumococcal isolates.
Abbreviation: NT, nontypeable.

Table 3.3: Distribution of age groups of patients with respect to penicillin susceptibility.

Age (year)[a]	Frequency (adjusted residual, % within age group, % within penicillin susceptibility group)													
	Susceptible				Intermediate				Resistant				Total	
< 5	20	(-1.2,	42.6,	26.7)	14	(0.1,	29.8,	31.8)	13	(1.3,	27.7,	40.6)	47	(31.1)
5 – 59	35	(0,	49.3,	46.7)	22	(0.5,	31.0,	50.0)	14	(-0.4,	19.7,	43.8)	71	(47.0)
≥ 60	20	(1.4,	60.6,	26.7)	8	(-0.7,	24.2,	18.2)	5	(-1,	15.2,	15.6)	33	(21.9)
Total	75	(49.7)			44	(29.1)			32	(21.2)			151	

[a]Chi-squared test found no significant association ($p = 0.524$) between age of patients and penicillin susceptibility of pneumococcal isolates (significant at $p \leq 0.05$).
Highlighted in yellow: The number (percentage) of penicillin-susceptible and –resistant pneumococcal isolates among children < 5 years old.
Highlighted in blue: The number (percentage) of penicillin-susceptible and –resistant pneumococcal isolates among elderly adults ≥ 60 years old.

Table 3.4: Distribution of age groups of patients with respect to sites of pneumococcal isolations.

Age[a]	Frequency (adjusted residual, % within age group, % within clinical sites)										
	Invasive			Noninvasive							Total
	Blood	CSF	Pleural fluid	Sputum	NP	Eye swab/pus	Ear swab/pus	Tracheal secretion	BAL	Other[b]	
< 5	7 (-1.8,14.9,18.9)	1 (0.6,2.1,50.0)	1 (0.1,2.1,33.3)	2 (-4.3,4.3,4.8)	28 (6.5,59.6,73.7)	0 (-1.2,0.0,0.0)	5 (1.6,10.6,55.6)	2 (-1.1,4.3,16.7)	1 (0.6,2.1,50.0)	0 (-1.2,0.0,0.0)	47 (31.1)
5 - 59	16 (-0.5,22.5,43.2)	1 (0.1,1.4,50.0)	2 (0.7,2.8,66.7)	27 (2.6,38.0,64.3)	10 (-3,14.1,26.3)	3 (1.9,4.2,100.0)	4 (-0.2,5.6,44.4)	6 (0.2,8.5,50.0)	0 (-1.3,0.0,0.0)	2 (0.7,2.8,66.7)	71 (47.0)
≥ 60	14 (2.7,42.4,37.8)	0 (-0.8,0.0,0.0)	0 (-0.9,0.0,0.0)	13 (1.7,39.4,31.0)	0 (-3.8,0.0)	0 (-0.9,0.0,0.0)	0 (-1.6,0.0,0.0)	4 (1.0,12.1,33.3)	1 (1.0,3.0,50.0)	1 (0.5,3.0,33.3)	33 (21.9)
Total	37(24.5)	2(1.3)	3(2.0)	42(27.8)	38(25.2)	3(2.0)	9(6.0)	12(7.9)	2(1.3)	3(2.0)	151

[a]Fisher's exact test found significant association ($p < 0.001$) between age of patients and clinical sites of isolations (significant at $p \leq 0.05$).
Adjusted residual value of $\geq +2$ or ≤ -2 was used to determine the underlying positively or negatively associated groups, respectively.
[b]Other sites include left tonsil, Bartholin's abscess, and vaginal discharge.
Highlighted in yellow: Pneumococci isolated from children aged < 5 were predominantly isolated from NP sites.
Highlighted in green: Pneumococci isolated from elderly adults ≥ 60 were predominantly isolated from blood.
Highlighted in purple: Sputum and NP represent the two main noninvasive sites of pneumococcal isolation.
Highlighted in blue: Blood represent the main invasive site of pneumococcal isolation.
Abbrevations: CSF, cerebrospinal fluid; NP, nasopharyngeal; BAL, bronchoalveolar lavage.

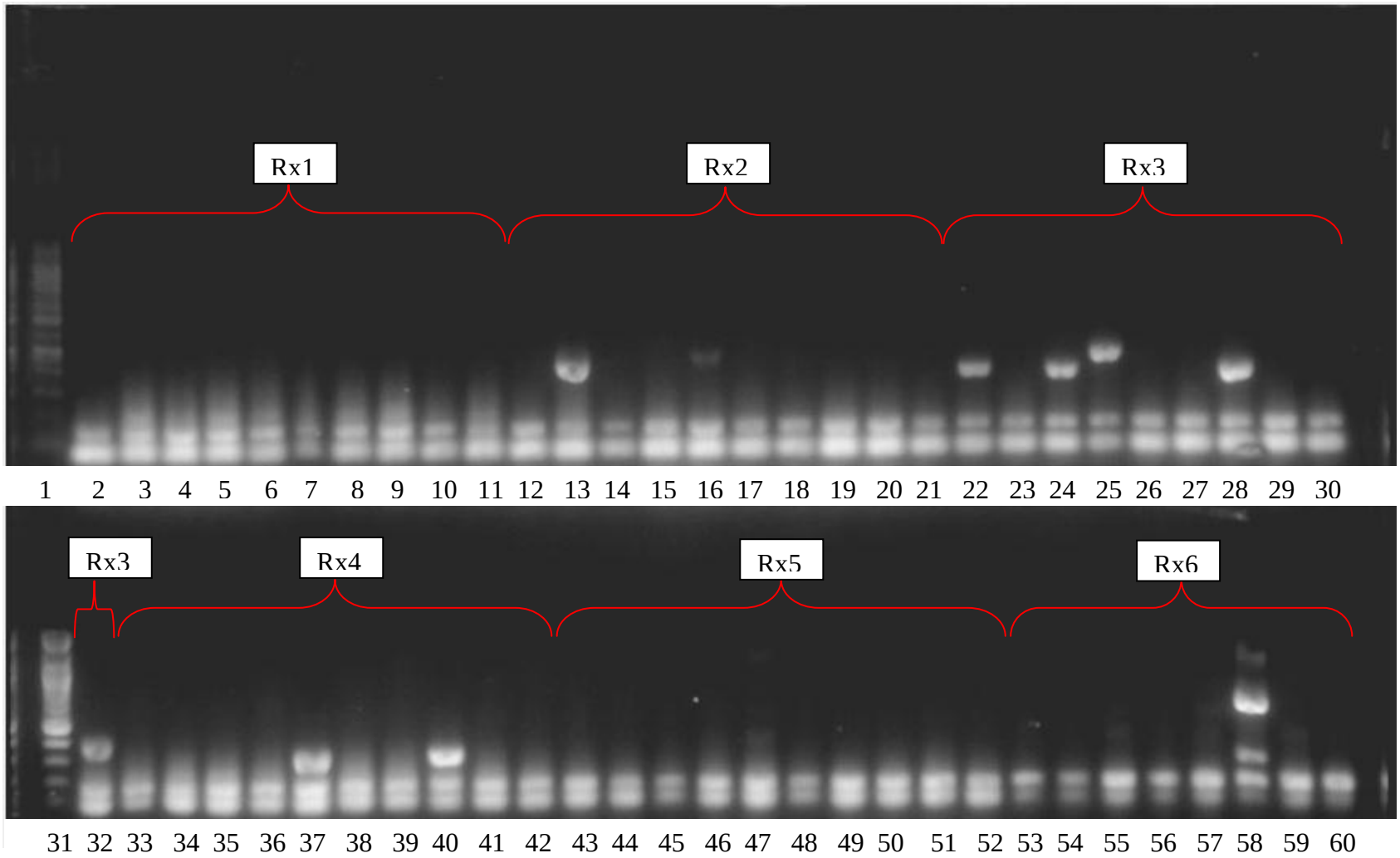


Figure 3.1: Representative complete seven multiplex reactions (Rx1 – Rx7) performed on 10 PRSP strains for serotyping.

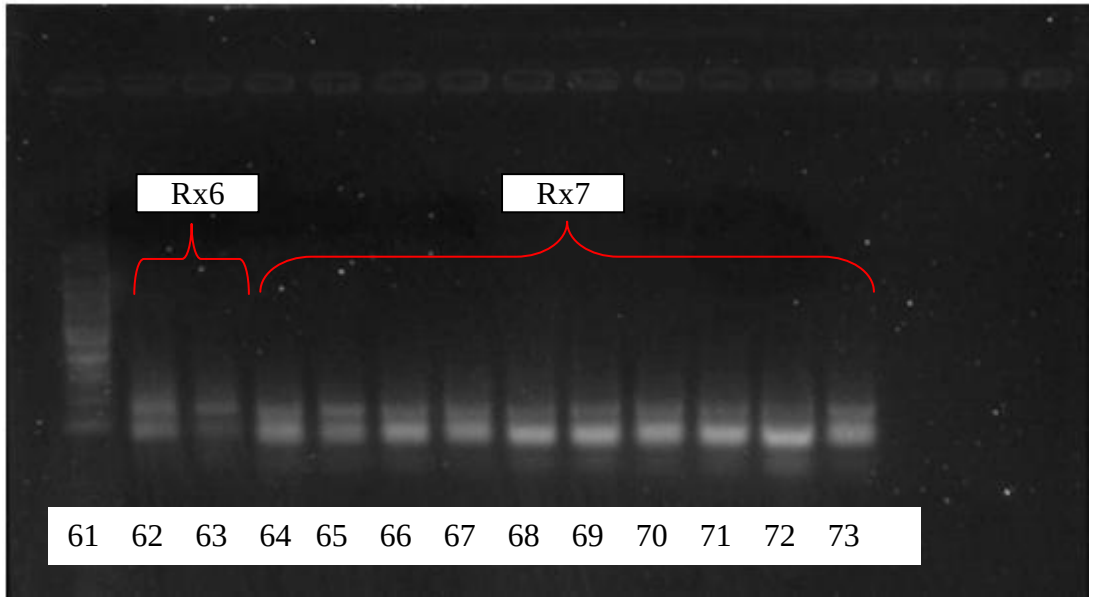


Figure 3.1(continued): Representative complete seven multiplex reactions (Rx1 – Rx7) performed on 10 PRSP strains for serotyping. Each reaction consisted of four different primers corresponding to the respective serotypes/serogroups as listed under Table 2.1. The strains were arranged in the order of (from left to right): S40(1), S41(12F), S44(19F), S46(11A/D), S47(12F), S48(1), S49(23F), S50(19F), S55(NT), and S56(23F) in each reaction (annotations in parenthesis denotes the serotype). Abbreviations: NT, Nontypeable; Rx, reaction.
Lane 2 – 11: Reaction 1, Strains S40, S41, S44, S46, S47, S48, S49, S50, S55, and S56.
Lane 12 – 21: Reaction 2, Strains S40, S41, S44, S46, S47, S48, S49, S50, S55, and S56.
Lane 22 – 30, 32: Reaction 3, Strains S40, S41, S44, S46, S47, S48, S49, S50, S55, and S56.
Lane 33 – 42: Reaction 4, Strains S40, S41, S44, S46, S47, S48, S49, S50, S55, and S56.
Lane 43 – 52: Reaction 5, Strains S40, S41, S44, S46, S47, S48, S49, S50, S55, and S56.
Lane 53 - 60, 62-63: Reaction 6, Strains S40, S41, S44, S46, S47, S48, S49, S50, S55, and S56.
Lane 64 – 73: Reaction 7, Strains S40, S41, S44, S46, S47, S48, S49, S50, S55, and S56.
Lane 1, 31, 61: 100kb DNA ladder.

Table 3.5: Pneumococcal serotypes distribution with respect to invasiveness of isolates.

Serotype[a]	Frequency (adjusted residual, % within serotype, % within invasiveness)											
	Invasive					Noninvasive					Total	
1	3	(0.4,	33.3,	7.1)		6	(-0.4,	66.7,	5.5)		9	(6.0)
3	0	(-0.6,	0.0,	0.0)		1	(0.6,	100.0,	0.9)		1	(0.7)
14	3	(1.6,	60.0,	7.1)		2	(-1.6,	40.0,	1.8)		5	(3.3)
34	0	(-0.9,	0.0,	0.0)		2	(0.9,	100.0,	1.8)		2	(1.3)
10A	0	(-0.6,	0.0,	0.0)		1	(0.6,	100.0,	0.9)		1	(0.7)
11A/11D	2	(1.5,	66.7,	4.8)		1	(-1.5,	33.3,	0.9)		3	(2.0)
12F/12A	2	(1.5,	66.7,	4.8)		1	(-1.5,	33.3,	0.9)		3	(2.0)
15A	0	(-0.6,	0.0,	0.0)		1	(0.6,	100.0,	0.9)		1	(0.7)
15B/15C	1	(1.6,	100.0,	2.4)		0	(-1.6,	0.0,	0.0)		1	(0.7)
16F	1	(0.7,	50.0,	2.4)		1	(-0.7,	50.0,	0.9)		2	(1.3)
sg18	0	(-0.9,	0.0,	0.0)		2	(0.9,	100.0,	1.8)		2	(1.3)
19A	6	(3.1,	75.0,	14.3)		2	(-3.1,	25,0,	1.8)		8	(5.3)
19F	6	(-3.6,	10.7,	14.3)		50	(3.6,	89.3,	45.9)		56	(37.1)
23F	4	(-0.3,	25.0,	9.5)		12	(0.3,	75.0,	11.0)		16	(10.6)
35B	1	(-0.1,	25.0,	2.4)		3	(0.1,	75.0,	2.8)		4	(2.6)
35F/47F	1	(0.2,	33.3,	2.4)		2	(-0.2,	66.7,	1.8)		3	(2.0)
6A/6B	1	(-1.2,	11.1,	2.4)		8	(1.2,	88.9,	7.3)		9	(6.0)
7C/7B/40	1	(1.6,	100.0,	2.4)		0	(-1.6,	0.0,	0.0)		1	(0.7)
7F/7A	1	(0.2,	33.3,	2.4)		2	(-0.2,	66.7,	1.8)		3	(2.0)
9V/9A	1	(1.6,	100.0,	2.4)		0	(-1.6,	0.0,	0.0)		1	(0.7)
NT	8	(1.3,	40.0,	19.0)		12	(-1.3,	60.0,	11.0)		20	(13.2)
Total	42	(27.8)				109	(72.2)				151	

[a]Fisher's exact test found significant association ($p = 0.001$) between serotypes and invasiveness of pneumococcal isolates (significant at $p \leq 0.05$). Adjusted residual value of $\geq +2$ or ≤ -2 was used to determine the underlying positively or negatively associated groups, respectively.

Highlighted in yellow: Serotypes 19F and 19A were associated with noninvasive and invasive pneumococcal isolates, respectively.

Abbreviation: NT, nontypeable

Table 3.6: Pneumococcal serotypes distribution with respect to age groups.

Serotype[a]	Frequency (adjusted residual, % within serotype, % within age group)												Total	
	< 5 years				5 – 59 years				≥ 60 years					
1	3	(0.1,	33.3,	6.4)	2	(-1.5,	22.2,	2.8)	4	(1.7,	44.4,	12.1)	9	(6.0)
3	0	(-0.7,	0.0,	0.0)	0	(-0.9,	0.0,	0.0)	1	(1.9,	100.0,	3.0)	1	(0.7)
14	3	(1.4,	60.0,	6.4)	0	(-2.1,	0.0,	0.0)	2	(1.0,	40.0,	6.1)	5	(3.3)
34	2	(2.1,	100.0,	4.3)	0	(-1.3,	0.0,	0.0)	0	(-0.8,	0.0,	0.0)	2	(1.3)
10A	0	(-0.7,	0.0,	0.0)	1	(1.1,	100.0,	1.4)	0	(-0.5,	0.0,	0.0)	1	(0.7)
11A/11D	0	(-1.2,	0.0,	0.0)	2	(0.7,	66.7,	2.8)	1	(0.5,	33.3,	3.0)	3	(2.0)
12F/12A	1	(0.1,	33.3,	2.1)	0	(-1.6,	0.0,	0.0)	2	(1.9,	66.7,	6.1)	3	(2.0)
15A	0	(-0.7,	0.0,	0.0)	1	(1.1,	100.0,	1.4)	0	(-0.5,	0.0,	0.0)	1	(0.7)
15B/15C	0	(-0.7,	0.0,	0.0)	0	(-0.9,	0.0,	0.0)	1	(1.9,	100.0,	3.0)	1	(0.7)
16F	0	(-1.0,	0.0,	0.0)	1	(0.1,	50.0,	1.4)	1	(1.0,	50.0,	3.0)	2	(1.3)
sg18	1	(0.6,	50.0,	2.1)	1	(0.1,	50.0,	1.4)	0	(-0.8,	0.0,	0.0)	2	(1.3)
19A	1	(-1.2,	12.5,	2.1)	6	(1.6,	75.0,	8.5)	1	(-0.7,	12.5,	3.0)	8	(5.3)
19F	22	(1.7,	39.3,	46.8)	26	(-0.1,	46.4,	36.6)	8	(-1.7,	14.3,	24.2)	56	(37.1)
23F	3	(-1.1,	18.8,	6.4)	12	(2.4,	75.0,	16.9)	1	(-1.6,	6.3,	3.0)	16	(10.6)
35B	2	(0.8,	50.0,	4.3)	2	(0.1,	50.0,	2.8)	0	(-1.1,	0.0,	0.0)	4	(2.6)
35F/47F	1	(0.1,	33.3,	2.1)	1	(-0.5,	33.3,	1.4)	1	(0.5,	33.3,	3.0)	3	(2.0)
6A/6B	1	(-1.3,	11.1,	2.1)	6	(1.2,	66.7,	8.5)	2	(0.0,	22.2,	6.1)	9	(6.0)
7C/7B/40	0	(-0.7,	0.0,	0.0)	1	(1.1,	100.0,	1.4)	0	(-0.5,	0.0,	0.0)	1	(0.7)
7F/7A	0	(-1.2,	0.0,	0.0)	1	(-0.5,	33.3,	1.4)	2	(1.9,	66.7,	6.1)	3	(2.0)
9V/9A	0	(-0.7,	0.0,	0.0)	0	(-0.9,	0.0,	0.0)	1	(1.9,	100.0,	3.0)	1	(0.7)
NT	7	(0.4,	35.0,	14.9)	8	(-0.7,	40.0,	11.3)	5	(0.4,	25.0,	15.2)	20	(13.2)
Total[b]	47	(31.1)			71	(47.0)			33	(21.9)			151	

[a]Fisher's exact test found significant association ($p = 0.03$) between serotypes and age of patients (significant at $p \leq 0.05$). Adjusted residual value of $\geq +2$ or ≤ -2 was used to determine the underlying positively or negatively associated groups, respectively.
Highlighted in yellow: The three major serotypes 19F, 23F, and 6A/6B detected among those aged 5 – 59 years old.
Highlighted in blue: Serotype 19F was the predominant serotype among children < 5 years old.
Abbreviation: NT, nontypeable.

3.2. Design of synthetic antimicrobial peptides

A total of 54 potential natural antimicrobial peptides (AMPs) were shortlisted under the Small Peptide Library. These natural AMPs were originally sourced from human, amphibian, mammals, fishes, arthropods, insects, arachnids, plants, and bacteria. Ten natural AMPs were selected (PDB ID: 1Q71, 1D9J, 1M4F, 1MA2, 1T51, 1PG1, 1X7K, 1HU5, 1VM4, 1HR1) with length ranging from 13 – 25 amino acids, net charge (NetC) of 0 to +8, charge density (ChD) of 0.00 – 0.45, hydrophobic ratio (HR) of 33% to 76%, total hydrophobic value (THV) of -19.5 – 10.1, and grand average of hydropathy (GRAVY) of -1.026 – 0.777 (Table 3.7). A total of 10 designed AMPs were then rationally designed by systematic residual modifications based on the template sequences of natural AMPs concerning the charge and hydrophobicity of the peptides (Table 3.7). All designed AMPs were designed to have positive charges or neutral since the AMPs collected under the Small Peptide Library were of similar properties and that in general cationic AMPs have greater antimicrobial potential.

The first series of the first group designed AMPs was generated based on residual substitutions without chain length alteration mainly by single (DAMP5 and DAMP6) or double amino acids substitutions (DAMP3 and DAMP7): DAMP5 was generated by single amino acid substitution of the highly hydrophobic residue Val^{11} of 1Q71 to Ser^{11} which reduced the HR and THV by 5 and lower GRAVY by 0.238; 1VM4 had reduced THV by 5.1 and GRAVY by 0.392 with Val^{6} to Trp^{6} substitution to generate DAMP6 while the C-terminal deamidation also reduced the NetC by 1; DAMP3 was highly cationic (+8) and highly hydrophilic (-18.2) generated by double consecutive amino acids substitutions at $Ile^{6,7}$ to Trp^{6} - Pro^{7} from 1HU5; Similarly, DAMP7 was highly

hydrophilic (HR = -12.7, THV = -0.977) due to $Ala^{3,10}$ substitutions to Trp^{3} - Arg^{10} which also increased the NetC by one unit.

The second series of the first group designed AMPs was redesigned based on residual modifications with chain length alterations. The designed AMPs were mainly truncated at the terminal side or constituted partial fragment of the template natural AMPs. DAMP1 was generated by hybridization of the ten amino acids N-terminal fragment of 1Q71 with nine amino acids C-terminal fragment of 1D9J, bridged by one Ser residue. DAMP2 was the large central fragment of 1M4F with two and three amino acids truncations at both N- and C-terminal and introduced with Gly^{10} substitution to the bulky residue Phe^{10} which greatly increased the hydrophobicity of the peptide. DAMP4 and DAMP9 were the central nine and eleven amino acids fragments of 1MA2 and 1PG1, respectively. Both have lowered positive charge and less hydrophilic than the respective templates. The N-terminus of 1T51 was truncated to the eight amino acids DAMP8 while maintaining the C-terminal amidation. In contrast, DAMP10 was the N-terminal fragment of 1X7K with Cys^{4}, Phe^{5}, and Cys^{8} substitutions to $Arg^{4,5}$ and Trp^{8}.

To further enhance the antipneumococcal activities of designed AMPs, three series of second group designed AMPs with 13 amino acids in length have been redesigned based on the selected DAMP6, DAMP7, and 1T51. DAMP6 and DAMP7 were the most potential designed AMPs among the first group peptides while 1T51 was the natural AMP with the highest antipneumococcal activity. The algorithm of BioModroid software automatically substituted the peptide with all 20 amino acids at each position and then calculated the compatibility scores based on both NetC and hydrophobicity for each of the modified peptide with the target protein sequence which is the PBPs. However, each

modification was limited to four amino acids substitution to prevent excessive alterations to the peptides. As looping the peptide sequence through the whole sequence of the huge PBPs protein could be computationally intensive and time-consuming, the most potential binding site (pocket) from each of the six PBPs were first identified by Metapocket 2.0 server. Modified peptides with the best compatibility scores for each PBP pocket were scored and ranked. Hence, for each peptide there were six scoring pairs (to each PBP pocket). These newly generated peptides were then combined under a master ranking which consisted of all the peptides and the respective scores. Three of the top scoring peptides were selected and synthesized for testing. These second group designed AMPs based on DAMP6, DAMP7, and 1T51 were referred to as DP6 (DP61, DP62, and DP63), DP7 (DP71, DP72, and DP73), and DP5 (DP51, DP52, and DP53), respectively and are listed under Table 3.7. The flow of the peptide design was illustrated in Figure 3.2.

A separate series of five hybrid peptides (DM1, DM2, DM3, DM4, and DM5) were generated by combining partial fragments of DAMP6 (GLFDIWKKLVSDF) and DAMP7 (ILWWKWAWWRWRR-NH_2). To design DM1, The first four residues at the N-terminus of DAMP6 (GLFD) was unaltered while the remaining fragment from the 5^{th} residue onwards (IWKKLVSDF) was replaced with fragment of same length at the homologous position of DAMP7 (KWAWWRWRR-NH_2). DM2, DM3, DM4, and DM5 were designed using the same approach by substituting the N-terminal fragments of DAMP6 beginning from the 6^{th}/7^{th} (produced identical sequences as both DAMP6 and DAMP7 have Trp at position 6), 8^{th}, 9^{th}, and 10^{th} residue with fragments of DAMP7 at the homologous positions, respectively (Figure 3.3). The four amino acids at the C-terminal were also unaltered in all five hybrid peptides.

Table 3.7: Physicochemical properties of the natural AMPs and designed AMPs.

Peptide	Sequences	Length	NetC[b]	ChD	HR	THV[c]	GRAVY[c]
Series with length alterations							
1Q71	GGAGHVPEYFVGIGTPISFYG	21	0	0.00	33	8.4	0.400
1D9J	KWKLFKKIGIGKFLHSAKKF[a]	20	+9	0.45	45	-6.2	-0.310
DAMP1	GGAGHVPEYFSKFLHSAKKF[a]	20	+5	0.25	35	-7.3	-0.365
1M4F	DTHFPICIFCCGCCHRSKCGMCCKT	25	+4	0.16	52	9.7	0.388
DAMP2	HFPICIFCCFCCHRSKCGMC	20	+4	0.20	65	19.2	0.960
1MA2	KWCFRVCYRGICYRRCR	17	+6	0.35	47	-8.8	-0.518
DAMP4	VCYRGICYR	9	+2	0.22	44	1.7	0.189
1T51	ILGKIWEGIKSLF[a]	13	+2	0.15	53	10.1	0.777
DAMP8	WEGIKSLF[a]	8	+1	0.13	50	1.6	0.200
DP51	ILGGDWEGIVLLF[a]	13	-1	-0.08	61	18.3	1.408
DP52	ILGGGWEGIVGLF[a]	13	0	0.00	53	17.2	1.323
DP53	ILGKILVHHKSLF[a]	13	+5	0.38	53	12.0	0.923
1PG1	RGGRLCYCRRRFCVCVGR	18	+6	0.33	44	-4.5	-0.250
DAMP9	CYCRRRFCVCV	11	+3	0.27	63	6.4	0.582
1X7K	RRWCFRVCYRGRFCYRKCR	19	+8	0.42	42	-19.5	-1.026
DAMP10	RRWRRRVWYRGRF	13	+7	0.54	30	-28.0	-2,154

Table 3.7 (continued): Physicochemical properties of the natural AMPs and designed AMPs.

Peptide	Sequences	Length	NetC[b]	ChD	HR	THV[c]	GRAVY[c]
Series without length alterations							
1HU5	KNLRRIIRKIIHIIKKYG	18	+8	0.44	38	-6.7	-0.372
DAMP3	KNLRRWPRKIIHIIKKYG	18	+8	0.44	33	-18.2	-1.011
1Q71	GGAGHVPEYFVGIGTPISFYG	21	0	0.00	33	8.4	0.400
DAMP5	GGAGHVPEYFSGIGTPISFYG	21	0	0.00	28	3.4	0.162
1VM4	GLFDIVKKLVSDF[a]	13	+1	0.08	53	10.1	0.777
DAMP6	GLFDIWKKLVSDF	13	0	0.00	53	5.0	0.385
DP61	GLFDTNKKKKSDF	13	+2	0.15	23	-18.6	-1.431
DP62	GTTDIWKKKKSDF	13	+2	0.15	23	-18.8	-1.446
DP63	GSKDKKKKLVSDF	13	+3	0.23	23	-17.7	-1.362
1HR1	ILAWKWAWWAWRR[a]	13	+4	0.31	76	-3.7	-0.285
DAMP7	ILWWKWAWWRWRR[a]	13	+5	0.38	69	-12.7	-0.977
DP71	PHWWKWAWWHHRR[a]	13	+7	0.54	46	-26.8	-0.977
DP72	KHWWKHDWWRWRR[a]	13	+7	0.54	38	-35.7	-2.746
DP73	ILWWLLAWWRWPH[a]	13	+3	0.23	76	3.9	0.300
Hybrid series							
DM1	GLFDKWAWWRWRR[a]	13	+4	0.31	53	-16.5	-1.269
DM2	GLFDIWAWWRWRR[a]	13	+3	0.23	61	-8.1	-0.623
DM3	GLFDIWKWWRWRR[a]	13	+4	0.31	53	-13.8	-1.062
DM4	GLFDIWKKWRWRR[a]	13	+5	0.38	46	-16.8	-1.292
DM5	GLFDIWKKLRWRR[a]	13	+5	0.38	46	-12.1	-0.931

[a]C-terminal amidation (-NH_2).
[b]Lys (K), Arg (R), His (H), and C-terminal amidation was assigned with +1 charge. Asp (D) and Glu (E) was assigned with -1 charge.
[c]Higher positive values denote higher hydrophobicity and vice versa.
Abbreviations: NetC, net charge; ChD, charge density; HR, hydrophobic ratio; THV, total hydrophobic value; GRAVY, Grand Average of Hydropathy.

Step 1: Align both sequences from N-terminal (position 1) and randomly substitute two residues (red).

P H W W K W A W W R W R R **peptide**
A S K M Y A N Y S W N M T K T G T T N Q D N M Q Y **pocket**

↓

Step 2: Calculate the scores for the direct pairs (red) and both neighboring pairs for each peptide residue simultaneously (illustrating only the direct (red) and neighboring pairs (blue) for His^2 and Ala^7 (Green))

P H W W K W A W W R W R R
A S K M Y A N Y S W N M T K T G T T N Q D N M Q Y

↓

Step 3: The same peptide sequence is shifted to position 2, 3, 4…etc and the same calculations are performed.

P H W W K W A W W R W R R ⋯▶
A S K M Y A N Y S W N M T K T G T T N Q D N M Q Y

↓

Step 4: Continue with substituting the remaining 19 X 19 amino acids combinations and perform the same procedures for each of the pocket sites.

↓

Step 5: All scores were ranked.

Pocket	ASKMYANYSWNMTKTGTTNQDNMQY
Peptide	PHWWKWAWWRWRR
Score	0.616227

↓

Step 6: The best scoring peptide (with two residual substitution) for the respective pocket site is now the template peptide.

↓

Step 7: Substitutions continued and limited to the other 11 residues. The altered residues from the round 1 design will not be disturbed.

P H W W K W A W W H H R R ⋯▶ **peptide**
A S K M Y A N Y S W N M T K T G T T N Q D N M Q Y **pocket**

↓

Step 8: The top 3 best scoring peptides with different pocket sites were selected.

Figure 3.2: The flow in generating the DP5, DP6, and DP7 of the second group designed AMPs based on double Y algorithm using BioModroid software.

Peptide	Sequence												
DAMP6	G	L	F	D	I	W	K	K	L	V	S	D	F
DAMP7	I	L	W	W	K	W	A	W	W	R	W	R	R

DM	Sequence												
DM1	G	L	F	D	K	W	A	W	W	R	W	R	R
DM2	G	L	F	D	I	W	A	W	W	R	W	R	R
DM3	G	L	F	D	I	W	K	W	W	R	W	R	R
DM4	G	L	F	D	I	W	K	K	W	R	W	R	R
DM5	G	L	F	D	I	W	K	K	L	R	W	R	R

Figure 3.3: The generation of the five hybrid peptide DM1 – DM5 via fragment hybridizations of DAMP6 and DAMP7.
Highlighted in yellow: Peptide sequence from DAMP6.
Highlighted in blue: Peptide sequence from DAMP7.

3.2.1. *In vitro* antimicrobial activity of peptides

3.2.1.1. Determination of peptides' MICs against *S. pneumoniae*

The minimum inhibitory concentrations (MICs) of the peptides were tested against 60 pneumococcal isolates which consisted of 20 isolates each for PSSP, PISP, and PRSP. These included the 10 natural AMPs (1Q71, 1D9J, 1M4F, 1MA2, 1T51, 1PG1, 1X7K, 1HU5, 1VM4, and 1HR1), 10 designed AMPs from the first group (DAMP1- DAMP10), and 14 designed AMPs from the second group (DP6 series: DP61 - DP63; DP7 series: DP71 - DP73; DP5 series: DP51 - DP53; Hybrid series: DM1 – DM5).

Among the 10 template natural AMPs, 1T51 was the most potent peptide which showed antipneumococcal activity of 15.63 – 250 µg/ml against all 60 pneumococcal isolates tested (Table 3.8). The antipneumococcal activity of seven other natural AMPs (1Q71, 1D9J, 1MA2, 1PG1, 1X7K, 1HU5, 1VM4) were considerably weak (overall effective percentage of 3.3% - 38.3%) while no detectable MIC in the range of concentrations tested were noted for 1M4F and 1HR1.

The first group of designed AMPs based on the natural AMPs can be divided into two series: with and without chain length alterations. For series without chain length alterations, DAMP7 was found to inhibit 45.0% (9/20) PRSP, 30.0% (6/20) PISP, and 65.0% (13/20) PSSP giving an overall effective percentage of 46.7% (28/60) in the effective range of 31.3 – 250 µg/ml (Table 3.8, highlighted in yellow). As compared to the template 1HR1 which was non-effective against *S. pneumoniae*, the antipneumococcal activity of DAMP7 was significantly higher. DAMP6 was rather weak showing high MIC level (125 – 250 µg/ml) against 30% of PRSP isolates only. The antipneumococcal activity of DAMP6 (10.0% overall effective percentage) was lowered as compared to the template 1VM4 (28.3% overall effective percentage). For

series with chain length alterations, no MIC was detected for the six designed AMPs (DAMP1, DAMP2, DAMP4, DAMP8, DAMP9, and DAMP10).

To generate peptides with improved antipneumococcal activity, three series of the second group designed AMPs were redesigned based on the selected DAMP6, DAMP7, and the natural AMP 1T51 (Table 3.8). While the effective ranges of both DP71 (PRSP, 62.5 – 250 µg/ml; PISP, 62.5 – 250 µg/ml; PSSP, 125 – 250 µg/ml) and DP72 (PRSP, 62.5 – 250 µg/ml; 125 – 250 µg/ml; PSSP, 62.5 – 250 µg/ml) of the DP7 series (Table 3.8, highlighted in green) were comparable to DAMP7 (PRSP, 62.5 – 250 µg/ml; 125 – 250 µg/ml; PSSP, 31.3 – 250 µg/ml) (Table 3.8, highlight in yellow) considering the respective penicillin susceptibility groups, both DP71 and DP72 displayed strong antipneumococcal activity which was approximately two-fold higher in overall effective percentage (DP71, 86.7%, DP72, 96.7%) as compared to DAMP7 (46.7%). The DP6 and DP5 series which were redesigned from DAMP6 and 1T51 had no antipneumococcal MIC reported.

The most remarkable result was observed with the hybrid series which consists of DM1 – DM5. In general, all five DMs displayed high potent antipneumococcal activity against all sixty pneumococcal isolates tested (100% overall effective percentage) irrespective of the penicillin susceptibility of the isolates (Table 3.8, highlighted in blue). These isolates included serotypes 19F, 23F, 6A/B, 11A/D, 15A/F, 16F, 22F/A, 3F, and the nontypeable pneumococci. The effective ranges of the five DMs were fairly similar for the respective PRSP, PISP, and PSSP and differed by no more than four-fold dilution. For each of the pneumococcal penicillin susceptibility groups, the effective ranges and thus the MICs of all five DMs were equal to or lower (except DM1 against PSSP) than DAMP7. This indicates that the antimicrobial activity of DMs were stronger than DAMP7. Among the five DMs, DM3 was the

most potent peptide with an overall effective range of 7.81 - 62.5 µg/ml. This peptide was also the most potent peptide against all three groups of PRSP, PISP, and PSSP. Of note, DM3 showed a particular sixteen-fold reduction in the lowest detectable MIC against PISP comparing to DAMP7. Overall, the antipneumococcal activity of DMs has been enhanced dramatically as compared to both DAMP6 and DAMP7, the templates used to generate the hybrids. All five DMs, DP71, DP72, and DAMP7 were water-soluble while DAMP6 has moderate water solubility.

Table 3.8: Minimum inhibitory concentrations of natural AMPs and designed AMPs against *S. pneumoniae*.

Peptide	PRSP			PISP			PSSP			Overall ER (µg/ml)	Overall EP (%)
	ER (µg/ml)	no. of isolates (EP %)		ER (µg/ml)	no. of isolates (EP %)		ER (µg/ml)	no. of isolates (EP %)			
Series with length alterations											
1Q71	250	9	(45.0)	250	5	(25.0)	>250	0	(0.0)	250	23.3
1D9J	125 - 250	4	(20.0)	125 – 250	2	(10.0)	>250	0	(0.0)	125 – 250	10.0
DAMP1	> 250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)		0.0
1M4F	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
DAMP2	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
1MA2	250	5	(25.0)	125 – 250	3	(15.0)	>250	0	(0.0)	125 – 250	13.3
DAMP4	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
1T51	31.3 – 125	20	(100.0)	31.3 – 250	20	(100.0)	15.63 – 125	20	(100.0)	15.63 - 250	100.0
DAMP8	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
DP51	250	1	(5.0)	>250	0	(0.0)	>250	0	(0.0)	250	1.7
DP52	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0
DP53	125	1	(5.0)	250	1	(5.0)	250	2	(10.0)	125 – 250	6.7
1PG1	>250	0	(0.0)	250	1	(5.0)	250	1	(5.0)	250	23.3
DAMP9	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
1X7K	62.5 – 250	3	(15.0)	62.5 – 250	2	(10.0)	125 – 250	2	(10.0)	62.5 – 250	11.7
DAMP10	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0

Table 3.8 (continued): Minimum inhibitory concentrations of natural AMPs and designed AMPs against *S. pneumoniae*.

Peptide	PRSP			PISP			PSSP			Overall ER (μg/ml)	Overall EP (%)
	ER (μg/ml)	no. of isolates (EP %)		ER (μg/ml)	no. of isolates (EP %)		ER (μg/ml)	no. of isolates (EP %)			
Series without length alterations											
1HU5	125 – 250	4	(20.0)	125 – 250	5	(25.0)	62.5 – 250	14	(70.0)	62.5 – 250	38.3
DAMP3	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
1Q71	250	9	(45.0)	250	5	(25.0)	>250	0	(0.0)	23.3	250
DAMP5	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
1VM4	125 – 250	5	(25.0)	62.5 – 250	6	(30.0)	125 – 250	6	(30.0)	62.5 – 250	28.3
DAMP6	250	6	(30.0)	>250	0	(0.0)	>250	0	(0.0)	250	10.0
DP61	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
DP62	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
DP63	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
1HR1	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
DAMP7	62.5 – 250	9	(45.0)	125 – 250	6	(30.0)	31.3 – 250	13	(65.0)	31.3 – 250	46.7
DP71	62.5 – 250	19	(95.0)	62.5 – 250	17	(85.0)	125 – 250	16	(80.0)	62.5 – 250	86.7
DP72	62.5 – 250	20	(20.0)	125 – 250	19	(95.0)	62.5 – 250	19	(95.0)	62.5 – 250	96.7
DP73	>250	0	(0.0)	>250	0	(0.0)	>250	0	(0.0)	-	0.0
Hybrid series											
DM1	31.3 – 125	20	(100.0)	31.3 – 125	20	(100.0)	62.5 – 250	20	(100.0)	31.3 – 250	100.0
DM2	15.63 – 250	20	(100.0)	7.81 – 250	20	(100.0)	15.63 – 250	20	(100.0)	7.81 – 250	100.0
DM3	15.63 – 62.5	20	(100.0)	7.81 – 62.5	20	(100.0)	7.81 – 62.5	20	(100.0)	7.81 – 62.5	100.0
DM4	31.3 – 125	20	(100.0)	15.63 – 125	20	(100.0)	15.63 – 125	20	(100.0)	15.63 – 125	100.0
DM5	31.3 – 125	20	(100.0)	15.63– 125	20	(100.0)	15.63 – 125	20	(100.0)	15.63 – 125	100.0

Highlighted in yellow: Antipneumococcal ER and EP of DAMP7 against PRSP, PISP, and PSSP, respectively.
Highlighted in green: Antipneumococcal ER and EP of DP71 and DP72 against PRSP, PISP, and PSSP, respectively.
Highlighted in blue: Overall ER and overall EP of DM1 – DM5.
Abbreviations: PRSP, penicillin-resistant *S. pneumoniae*; PISP, penicillin-intermediate *S. pneumoniae*; PSSP, penicillin-susceptible *S. pneumoniae*; ER, effective range; EP, effective percentage.

3.2.1.2. Broad spectrum antibacterial activity of peptides

The second group designed AMPs together with DAMP6, DAMP7, and 1T51 were further tested using broth microdilution technique against eight bacterial species commonly encountered in the clinical setting to assess the spectrum of antibacterial activity of the peptides. The bacteria included *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 15442, *Acinetobacter baumanii* ATCC 15308, and one clinical isolate for each methicillin-resistant *S. aureus* (MRSA), *Enterococcus cloacae, Citrobacter* spp., and *K. pneumoniae*. DAMP7 was found to display antibacterial activity against the gram-positive *S. aureus* (62.5 µg/ml) and MRSA (31.25 µg/ml) but was weakly effective (125 - 250 µg/ml) against *E. coli*, *A. baumanii*, and *E. cloacae* except *Citrobacter* spp. (62.5 µg/ml) (Table 3.9, highlighted in yellow). No antibacterial activity was observed for DAMP6 and the redesigned DP6 series peptides.

For the DP7 series, DP71 was non-effective (≥125 µg/ml) while DP72 displayed lower antibacterial MICs in the range of 31.3 – 125 µg/ml (except for *K. pneumoniae*) than DP71. As compared to DAMP7, DP72 exhibited four-fold lower in antiMRSA activity (DAMP7, 31.3 µg/ml; DP72, 125 µg/ml), however, the antipseudomonal activity was more than four-fold higher (DAMP7, > 250 µg/ml; DP72, 62.5 µg/ml). Antibacterial activities other than these two bacteria were comparable by no more than two-fold difference in MICs. Apart from that, DP72 displayed the highest antibacterial activity against *Citrobacter spp.* (31.25 µg/ml) among the panel of bacteria tested. Despite DP73 was non-active against any of the bacteria tested, it showed a potent activity specifically against *S. aureus* at very low MIC level (7.81 µg/ml).

The hybrid DMs which were strongly effective against *S. pneumoniae* also displayed antibacterial activity against multiple bacteria. DM3 exhibited potent antibacterial activity in the range of 7.81 – 62.5 µg/ml against seven out of the eight bacteria tested except for *P. aeruginosa* (125 µg/ml) (Table 3.9, highlighted in blue). Notably, the DM3-susceptible strain included *K. pneumoniae* (DM3 MIC = 62.5 µg/ml) whereby *K. pneumoniae* was not susceptible (MIC > 250 µg/ml) to as many as 14 of the total peptides tested in this study. Furthermore, DM3 also showed enhanced broad spectrum activities as compared to DAMP7 by at least four-fold reduction in antibacterial MICs against all the eight bacteria.

While DM4 exhibited comparable antibacterial activity to DAMP7, DM1 and DM5 showed similar (against *E. cloaceae*) or at least two-fold reduction in MICs against gram-negative bacteria as compared to DAMP7. In addition, DM5 showed drastic improvement in antibacterial activity as compared to DAMP7; for *E. coli* and *P. aeruginosa*, the MICs were at least eight-fold lower (*E. coli*, DAMP7 MIC = 250 µg/ml, *P. aeruginosa*, DAMP7 MIC = > 250 µg/ml; DM5, 31.3 µg/ml for both); and for *A. baumanii*, the MIC was four-fold lower (DAMP7 MIC = 125 µg/ml; DM5 MIC = 31.3 µg/ml). DM1 also showed four-fold lower in MICs against *E. coli* (DAMP7 MIC = 250 µg/ml, DM1 MIC = 62.5 µg/ml) and *P. aeruginosa* (DAMP7 MIC = > 250 µg/ml, DM1 MIC = 125 µg/ml). However, the MICs of DM1 and DM5 were two-fold higher (125 µg/ml and 62.5 µg/ml) than DAMP7 (62.5 µg/ml and 31.3 µg/ml) against the gram-positive bacteria *S. aureus* and MRSA, respectively. Although the MICs against *S. aureus* and MRSA were comparable, the susceptibility of MRSA to DM1, DM3, and DM5 was two-fold higher than *S. aureus*. In contrast, the opposite was observed for DM2 whereby the susceptibility of MRSA was two-fold lower than *S. aureus*. It was noticed that the effective ranges of DAMP7 and hybrid DMs fell

closely within the overall effective range as that for *S. pneumoniae*. This implies that the antipneumococcal effective ranges of DAMP7 and hybrid DMs were also effective against wide variety of bacterial species.

Table 3.9: Broad spectrum antibacterial testing of peptides against eight common human bacterial pathogens.

Peptide	MIC (µg/ml)							
	Gram-positive		Gram-negative					
	S. aureus	MRSA	*E. coli*	*P. aeruginosa*	*A. baumanii*	*E. cloaceae*	*Citrobacter spp.*	*K. pneumoniae*
DAMP6	>250	>250	>250	>250	>250	>250	>250	>250
DP61	>250	>250	>250	>250	>250	>250	250	>250
DP62	>250	>250	>250	>250	>250	>250	>250	>250
DP63	>250	>250	>250	>250	>250	>250	>250	>250
DAMP7	62.5	31.3	250	>250	125	125	62.5	>250
DP71	>250	>250	>250	>250	125	>250	>250	>250
DP72	125	125	125	62.5	125	125	31.3	>250
DP73	7.81	>250	>250	>250	>250	>250	>250	>250
1T51	31.3	31.3	125	>250	31.3	250	125	125
DP51	>250	>250	>250	>250	>250	>250	>250	>250
DP52	>250	>250	>250	>250	>250	>250	>250	>250
DP53	>250	>250	>250	>250	>250	>250	>250	>250
Hybrid DM								
DM1	125	62.5	62.5	125	62.5	125	31.3	>250
DM2	31.3	62.5	250	>250	31.3	>250	62.5	>250
DM3	15.63	7.81	62.5	125	15.63	31.3	15.63	62.5
DM4	62.5	62.5	62.5	31.3	31.3	250	31.3	>250
DM5	125	62.5	31.3	31.3	31.3	125	31.3	250

Highlighted in yellow: MICs of DAMP7 against the eight gram-positive and gram-negative bacteria tested.
Highlighted in blue: MICs of DM3 against the eight gram-positive and gram-negative bacteria tested.
Abbreviations: MIC, minimum inhibitory concentrations

3.2.1.3. *In vitro* synergism activity

The *in vitro* synergism effects of peptide-peptide and peptide-penicillin combinations were tested using the checkerboard method on selected peptides with antipneumococcal activity including DAMP6, DAMP7, DP71, DP72, and the five DMs. Combination of peptide-peptide pairs at differing ratios were prepared in 96-well round bottom plates followed by addition of bacterial suspensions as described under section 2.5.4. Fractional inhibitory concentration (FIC) index of ≤ 0.05 denotes synergism, > 0.5 – 4 denotes additive/indifference, and > 4 denotes antagonism (Bajaksouzian *et al.*, 1996). Results showed that DAMP7, DP71, DP72, and the five hybrids all displayed FIC indexes of ≤ 0.5 when given in combination with penicillin against PRSP, PISP, and PSSP (Table 3.10, Table 3.11, and Table 3.12, highlighted in yellow), noting antipneumococcal synergism irrespective of penicillin susceptibility of the isolates. The FIC range determined was between 0.16 – 0.5. DAMP6 was tested against PRSP only as no PISP or PSSP were susceptible to this peptide (Table 3.8). No synergism was detected for DAMP6 and penicillin pair against PRSP. Among all the peptide-peptide combinations, synergism was noted when DM5 was paired with DM1, DM2, DM4, or DAMP7 (Table 3.10, highlighted in green) and when DM3 was paired with DAMP7 (Table 3.10, highlighted in blue) against PRSP. Other peptide-peptide combinations against PRSP as well as PISP and PSSP detected no synergism with FICs in the range of > 0.5 – 1.25. They were still able to inhibit pneumococci in an additive/indifference fashion implying that these components in combination had no significant interaction between them to enhance the antimicrobial activity of one another (Meletiadis *et al.*, 2010). No antagonism (FIC > 4) was detected in any of the pairs.

Table 3.10: FIC index of peptide-peptide/penicllin combinations against PRSP.

Peptide pair		FIC index	Interpretation[a]
DM1	DM2	0.75	Additive/indifference
	DM3	0.75	Additive/indifference
	DM4	0.75	Additive/indifference
	DM5	0.50	Synergy
	DAMP6	1.00	Additive/indifference
	DAMP7	0.75	Additive/indifference
	DP71	1.25	Additive/indifference
	DP72	1.25	Additive/indifference
	PEN	0.38	Synergy
DM2	DM3	0.75	Additive/indifference
	DM4	0.75	Additive/indifference
	DM5	0.50	Synergy
	DAMP6	1.00	Additive/indifference
	DAMP7	0.75	Additive/indifference
	DP71	1.25	Additive/indifference
	DP72	1.00	Additive/indifference
	PEN	0.38	Synergy
DM3	DM4	0.75	Additive/indifference
	DM5	0.75	Additive/indifference
	DAMP6	1.00	Additive/indifference
	DAMP7	0.50	Synergy
	DP71	1.25	Additive/indifference
	DP72	1.25	Additive/indifference
	PEN	0.38	Synergy
DM4	DM5	0.50	Synergy
	DAMP6	1.25	Additive/indifference
	DAMP7	0.75	Additive/indifference
	DP71	1.25	Additive/indifference
	DP72	1.25	Additive/indifference
	PEN	0.28	Synergy
DM5	DAMP6	1.25	Additive/indifference
	DAMP7	0.50	Synergy
	DP71	0.75	Additive/indifference
	DP72	1.00	Additive/indifference
	PEN	0.25	Synergy
DAMP6	DAMP7	1.25	Additive/indifference
	DP71	1.25	Additive/indifference
	DP72	1.25	Additive/indifference
	PEN	1.00	Additive/indifference
DAMP7	DP71	1.06	Additive/indifference
	DP72	1.06	Additive/indifference
	PEN	0.31	Synergy
DP71	DP72	1.25	Additive/indifference
	PEN	0.19	Synergy
DP72	PEN	0.38	Synergy

[a]FIC ≤ 0.05 denotes synergism, > 0.5 – 4 denotes indifference, and > 4 denotes antagonism.
Combinations showing *in vitro*antipneumococcal synergism:
Highlighted in yellow: Penicillin-DM1/DM2/DM3/DM4/DM5/DAMP7/DP71/DP72.
Highlighted in green: DM5–DM1/DM2/DM4/DM7
Highlighted in blue: DM3-DAMP7.
Abbreviation: FIC, fractional inhibitory concentration.

Table 3.11: FIC index of peptide-peptide/penicllin combinations against PISP.

Peptide pair		FIC index	Interpretation[a]
DM1	DM2	0.75	Additive/indifference
	DM3	0.75	Additive/indifference
	DM4	0.63	Additive/indifference
	DM5	0.63	Additive/indifference
	DAMP6	NE	
	DAMP7	0.75	Additive/indifference
	DP71	1.00	Additive/indifference
	DP72	1.25	Additive/indifference
	PEN	0.50	Synergy
DM2	DM3	0.75	Additive/indifference
	DM4	0.63	Additive/indifference
	DM5	0.63	Additive/indifference
	DAMP6	NE	
	DAMP7	1.00	Additive/indifference
	DP71	1.00	Additive/indifference
	DP72	1.00	Additive/indifference
	PEN	0.50	Synergy
DM3	DM4	0.63	Additive/indifference
	DM5	0.75	Additive/indifference
	DAMP6	NE	
	DAMP7	1.00	Additive/indifference
	DP71	1.00	Additive/indifference
	DP72	1.25	Additive/indifference
	PEN	0.50	Synergy
DM4	DM5	0.75	Additive/indifference
	DAMP6	NE	
	DAMP7	0.75	Additive/indifference
	DP71	1.25	Additive/indifference
	DP72	1.00	Additive/indifference
	PEN	0.50	Synergy
DM5	DAMP6	NE	
	DAMP7	0.75	Additive/indifference
	DP71	1.00	Additive/indifference
	DP72	1.00	Additive/indifference
	PEN	0.50	Synergy
DAMP6	DAMP7	NE	
	DP71	NE	
	DP72	NE	
	PEN	NE	
DAMP7	DP71	1.00	Additive/indifference
	DP72	1.00	Additive/indifference
	PEN	0.31	Synergy
DP71	DP72	1.25	Additive/indifference
	PEN	0.50	Synergy
DP72	PEN	0.50	Synergy

[a]FIC ≤ 0.05 denotes synergism, > 0.5 – 4 denotes indifference, and > 4 denotes antagonism.
Combinations showing *in vitro*antipneumococcal synergism:
Highlighted in yellow: Penicillin-DM1/DM2/DM3/DM4/DM5/DAMP7/DP71/DP72.
Abbreviations: FIC, fractional inhibitory concentration; NE, Not tested

Table 3.12: FIC index of peptide-peptide/penicllin combinations against PSSP.

	Peptide pair	FIC index	1.10. Interpretation[a]
DM1	DM2	0.63	Additive/indifference
	DM3	0.63	Additive/indifference
	DM4	0.63	Additive/indifference
	DM5	0.63	Additive/indifference
	DAMP6	NE	
	DAMP7	0.63	Additive/indifference
	DP71	1.00	Additive/indifference
	DP72	1.25	Additive/indifference
	PEN	0.16	Synergy
DM2	DM3	1.13	Additive/indifference
	DM4	1.13	Additive/indifference
	DM5	0.63	Additive/indifference
	DAMP6	NE	
	DAMP7	1.13	Additive/indifference
	DP71	1.00	Additive/indifference
	DP72	1.25	Additive/indifference
	PEN	0.25	Synergy
DM3	DM4	1.13	Additive/indifference
	DM5	1.13	Additive/indifference
	DAMP6	NE	
	DAMP7	1.13	Additive/indifference
	DP71	1.00	Additive/indifference
	DP72	1.00	Additive/indifference
	PEN	0.19	Synergy
DM4	DM5	0.63	Additive/indifference
	DAMP6	NE	
	DAMP7	0.63	Additive/indifference
	DP71	1.00	Additive/indifference
	DP72	1.25	Additive/indifference
	PEN	0.28	Synergy
DM5	DAMP6	NE	
	DAMP7	1.13	Additive/indifference
	DP71	1.25	Additive/indifference
	DP72	1.25	Additive/indifference
	PEN	0.19	Synergy
DAMP6	DAMP7	NE	
	DP71	NE	
	DP72	NE	
	PEN	NE	
DAMP7	DP71	1.00	Additive/indifference
	DP72	1.00	Additive/indifference
	PEN	0.19	Synergy
DP71	DP72	1.25	Additive/indifference
	PEN	0.38	Synergy
DP72	PEN	0.38	Synergy

[a]FIC ≤ 0.05 denotes synergism, > 0.5 – 4 denotes indifference, and > 4 denotes antagonism.
Combinations showing *in vitro*antipneumococcal synergism:
Highlighted in yellow: Penicillin-DM1/DM2/DM3/DM4/DM5/DAMP7/DP71/DP72.
Abbreviations: FIC, fractional inhibitory concentration; NE, Not tested

3.2.1.4. Assessing the killing kinetics of peptides against *S. pneumoniae*

Based on the MIC results, peptides displaying antipneumococcal activity were assayed against each PRSP, PISP, and PSSP isolate to determine the killing kinetics over time posttreatment. Penicillin as the commonly used antibiotic against *S. pneumoniae* was also included as standard antibiotic control to compare the pneumocidal activity of peptides. Surviving colony forming units (CFU) at 0 (pretreatment), 30, 60, 120, 150, 180, 210, and 240 mins posttreatment were enumerated and expressed as percentage of cells recovered (%) over untreated control at the respective time points.

DAMP7 showed strong and rapid pneumocidal effects noting complete clearances (100%) of PRSP and PISP and near complete clearance (96.9%) of PSSP immediately at 30 mins posttreatment. This killing kinetics was greater than penicillin by 61.1%, 77.8%, and 57.2% against PRSP, PISP, and PSSP, respectively at an average of 65.4% (Figure 3.4). In contrast, although comparable PRSP killing rates were observed for DAMP6 (59.2%) and penicillin (64.1%) at first 30 mins posttreatment, the killing rate over prolonged period were lower for DAMP6 than penicillin (Figure 3.4A). As at 150 mins posttreatment, 21.9% of DAMP6-treated PRSP was recovered while only 4.8% of penicillin-treated PRSP was recovered. DAMP6 was not assayed against PISP and PSSP as it has no detectable MICs against these groups of pneumococci.

The redesigned DP71 and DP72 also displayed strong killing effects. The percentages of DP71-treated cells recovered at 30 mins posttreatment was 22.2%, 21.9%, and 15.3% at an average of 19.8% while that of DP72-treated cells were 43.7%, 32.2%, and 89.9% at an average of 55.3% for PRSP, PISP, and PSSP, respectively at 30 mins posttreatment (Figure 3.5). In comparison, the differences in

killing rates over penicillin against PRSP, PISP, and PSSP were 41.9%, 55.9%, and 45.0% respectively at an average of 47.6% for DP71; 20.4%, 45.6%, and 29.6% at an average of 31.8% for DP72.

Drastic reductions in viable pneumococci were also noted with the five DMs (Figure 3.6). Pneumocidal activity of the peptides at 30 mins posttreatment showed that while the proportions of penicillin-treated pneumococcal cells recovered remained at 60 – 78%, the percentages of DMs-treated pneumococci recovered were less than 30% irrespective of the penicillin susceptibility of the isolates. This indicates that although the pneumocidal activity varied among the respective DMs, large proportions of the pneumococcal cells was rapidly cleared within the first 30 mins and the CFU recovered continue to drop until eventual elimination. The pneumocidal activities of DMs were dramatically greater than penicillin. The differences in killing kinetics against PRSP, PISP, and PSSP over penicillin were 52.9%, 54.2%, and 38.4% respectively at an average of 48.5% for DM1; 64.1%, 65.4%, and 54.4% respectively at an average of 61.3% for DM2; 60.0%, 76.0%, and 57.7% respectively at an average of 64.6% for DM3; 39.0%, 57.8%, and 30.5% respectively at an average of 42.4% for DM4; 38.1%, 58.5%, and 36.5% respectively at an average of 44.4% for DM5. The results clearly showed that the hybrids were superior to penicillin in pneumocidal activities. Notably, the proportions of surviving DM2- and DM3-treated cells were < 6.0% for all three penicillin susceptible pneumococci at 30 min posttreatment (except 12.4% by DM2-treated PISP). Moreover, complete bacterial clearance was observed with DM2-treated PRSP immediately at the first 30 mins posttreatment.

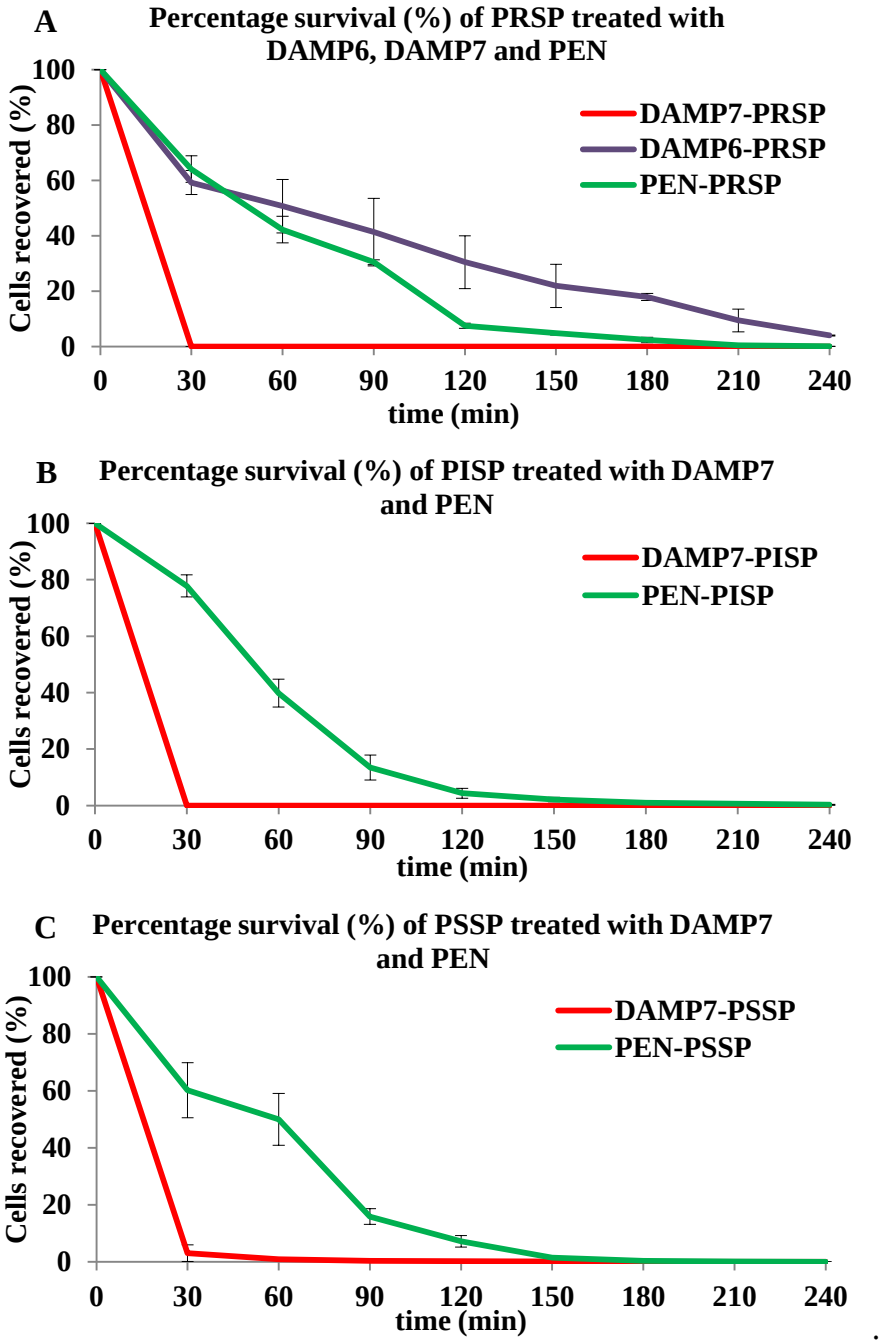


Figure 3.4: Percentage (%) of pneumococcal cells recovered following treatment with DAMP6, DAMP7, and PEN. At the specific time intervals, each (A) PRSP, (B) PISP, and (C) PSSP at $5x10^6$ CFU/ml were treated with 2X MIC of the respective peptides/PEN and viable cells posttreatment were enumerated using plate count method. DAMP7 was strongly pneumocidal at an average of 65.4% as compared to PEN at 30 min posttreatment. The killing kinectic of DAMP6 against PRSP was lower than PEN over prolonged treatment time.

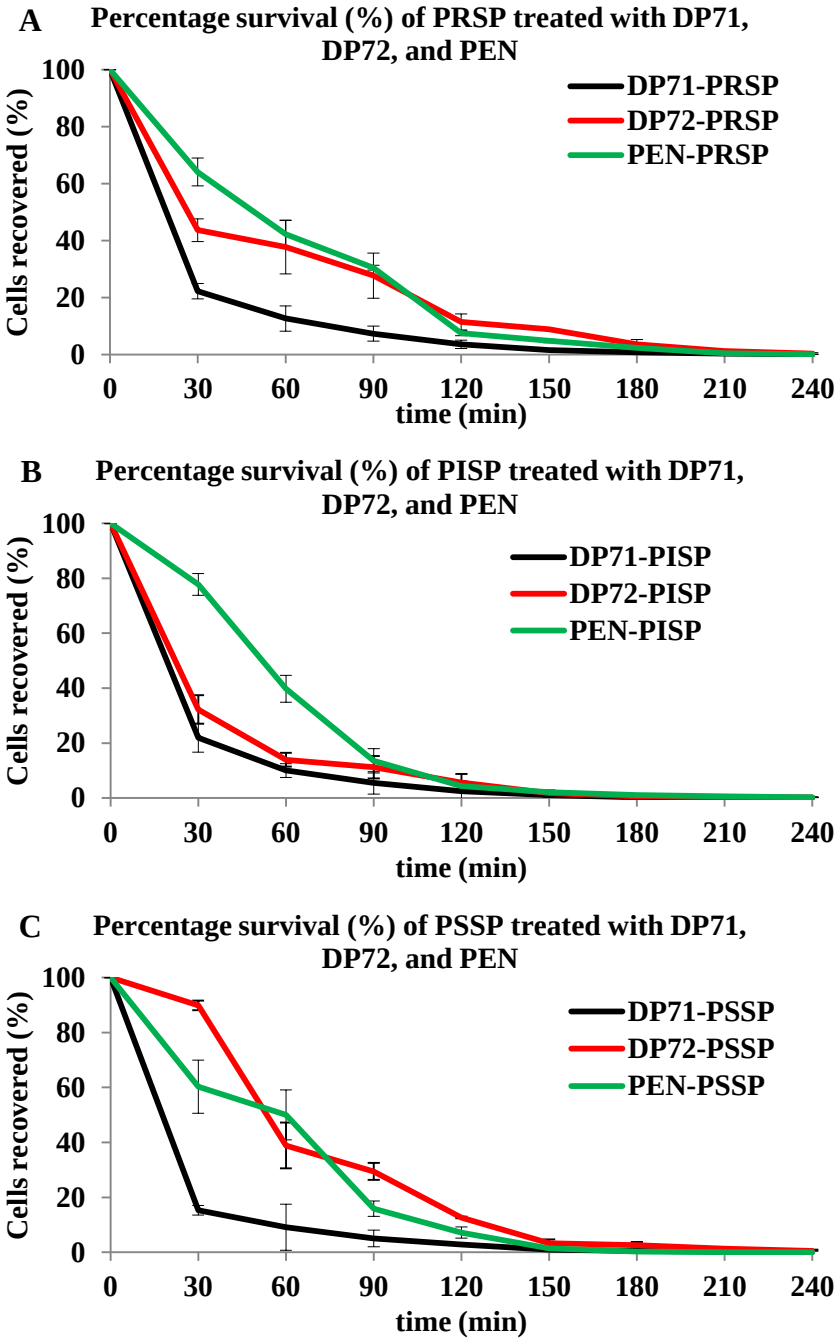


Figure 3.5: Percentage (%) of pneumococcal cells recovered following treatment with DP71, DP72, and PEN. At the specific time intervals, each (A) PRSP, (B) PISP, and (C) PSSP at $5x10^6$ CFU/ml were treated with 2X MIC of the respective peptides/PEN and viable cells posttreatment were enumerated using plate count method. DP71 was pneumocidal at an average of 47.6% higher killing rate than PEN at 30 min posttreatment while that of DP72 was 31.8% although the pneumocidal activity of DP72 against PRSP and PSSP was fairly comparable to PEN.

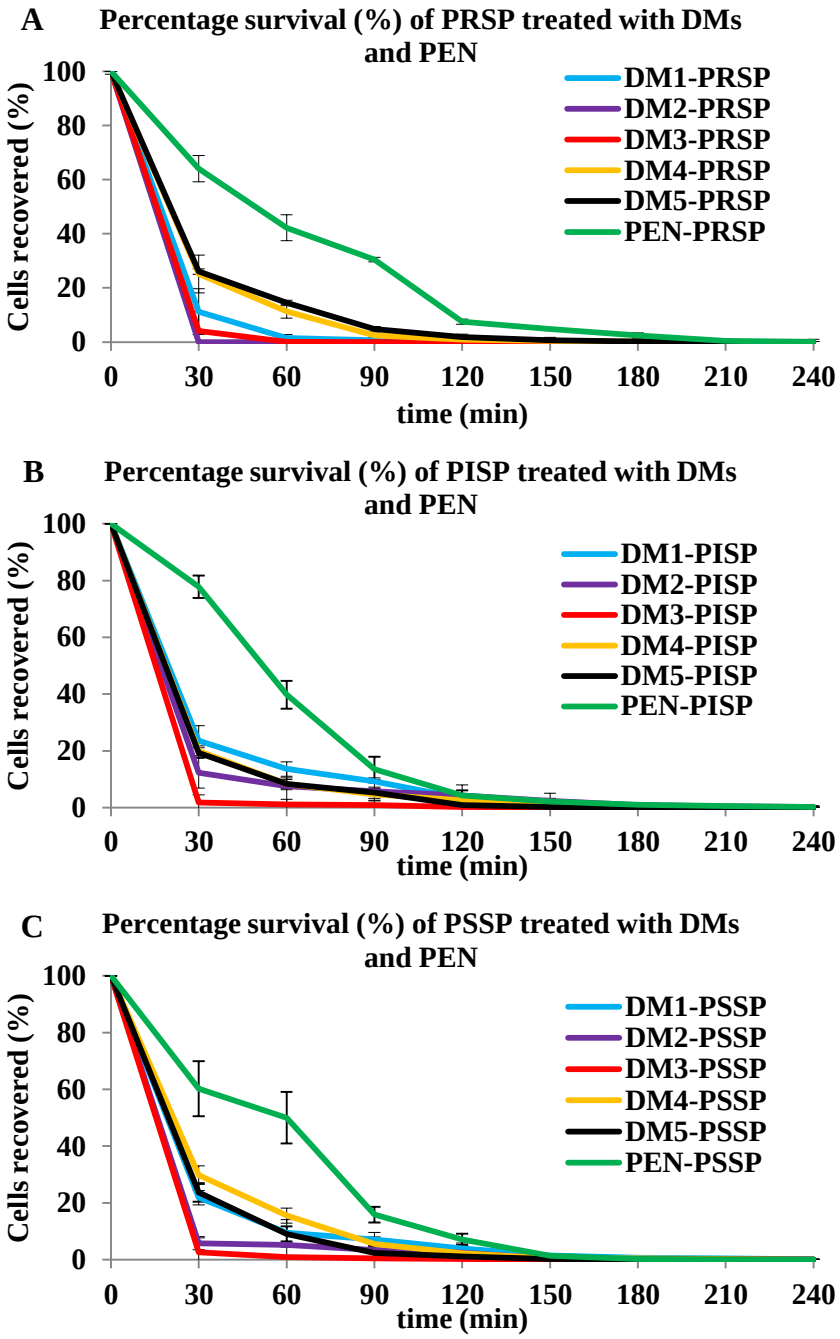


Figure 3.6: Percentage (%) of pneumococcal cells recovered following treatment with DM1 – DM5 and PEN. At the specific time intervals, each (A) PRSP, (B) PISP, and (C) PSSP at $5x10^6$ CFU/ml were treated with 2X MIC of the respective peptides/PEN and viable cells posttreatment were enumerated using plate count method. The five DMs (DM1 – DM5) were strongly pneumocidal at an average of 48.5%, 61.3%, 64.6%, 42.4%, and 44.4% higher killing rates than PEN at 30 min posttreatment, respectively.

3.2.1.5. Effects of peptides on pneumococcal cell morphology

The changes in cell morphology induced by the five DMs on *S. pneumoniae* were investigated using Transmission Electron Microscopy (TEM). As shown in Figure 3.7A, the untreated cell has intact cell wall and cytoplasmic membrane which maintain the coccoidal shape of pneumococcus. The cytoplasm was densely packed and the pneumococcal cell surface structures especially the capsular polysaccharide (CPS) were visible as a thin layer covering the outer layer of the cell (Figure 3.7A, black arrow). Following treatment with each DM, the pneumococcal cells were extensively enlarged and/or became spherical (Figure 3.7B - K). Severe damages accompanied with overwhelming morphological changes of the pneumococcal cells were clearly evident. In particular, cell wall and/or cytoplasmic membrane breakages of small/large magnitudes were observed (arrow a). Losses in large areas of the cell wall were noted especially with DM4- and DM5-treated pneumococci which appeared as "balding" thus exposing the cytoplasmic membrane of cells (arrow a1). Detachment of the cell wall can be clearly seen, forming large cavity spaces between the cell wall and cytoplasmic membrane (arrow b). With the loss of the structural support from cell wall and the weakened cytoplasmic membrane, the cells become extensively irregular in shape. Leakage of intracellular contents eventually occurred (arrow c). As in DM3, the release of cytoplasmic contents seems to happen through the breakdown and subsequent formation of cytoplasmic-containing inclusion bodies (arrow d, Figure 3.7B, G, K) or through bulging of cytoplasmic membrane leading to formation of inclusion bodies as with DM5 (arrow d, Figure 3.7J). Additionally, large halos observed in the cytoplasm could probably be due to loss of cytoplasmic content (arrow e). For DM2, some intracellular content of the treated cell also appeared filamentous and stacked at one pole of the cell (Figure 3.7D, arrow f) as compared to

the non-filamentous cytoplasm distributed mainly in the center region of the cell. Eventually cells without cellular content appeared as an empty “shell”.

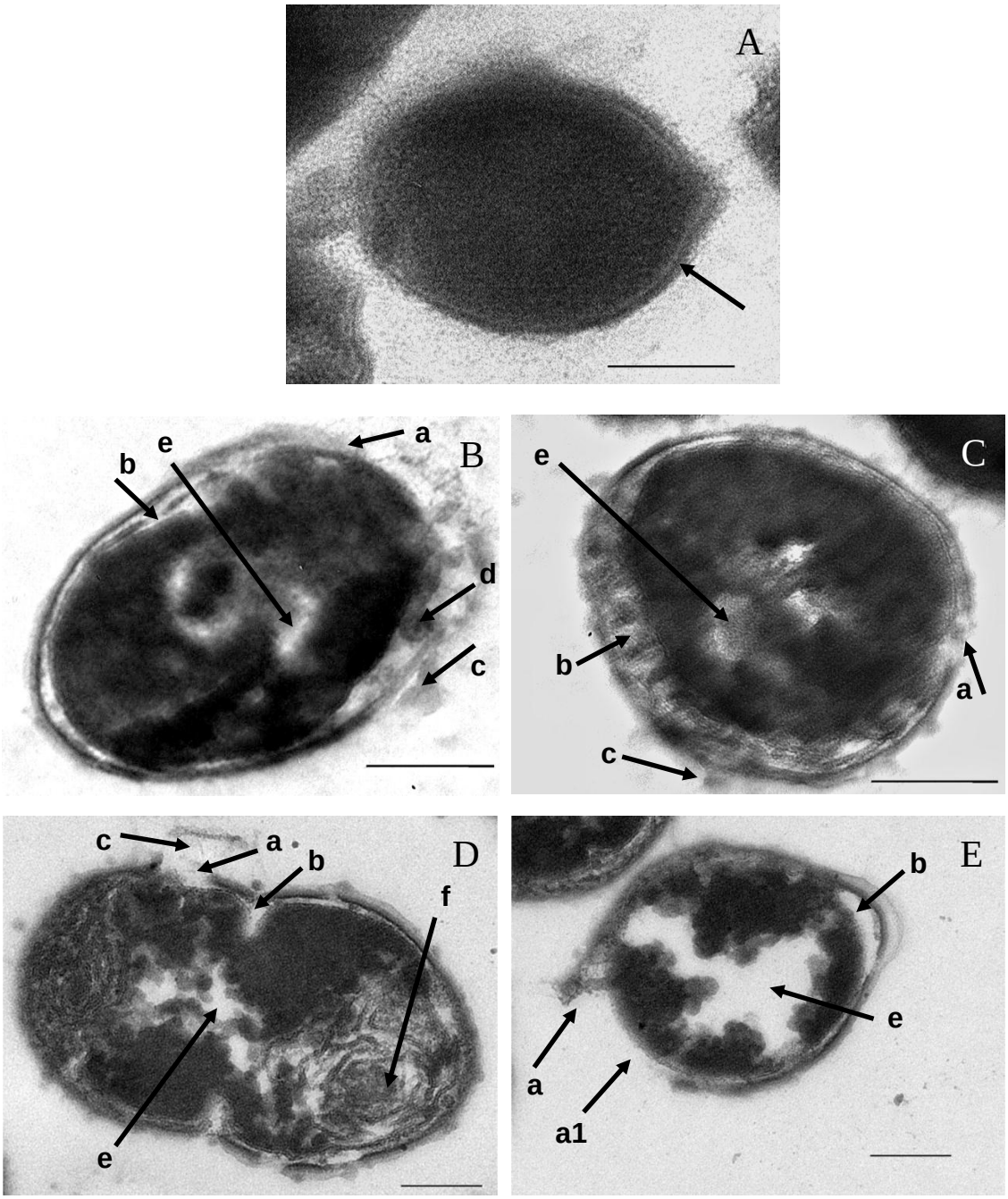


Figure 3.7: TEM images of PRSP treated with DM1 – DM5.

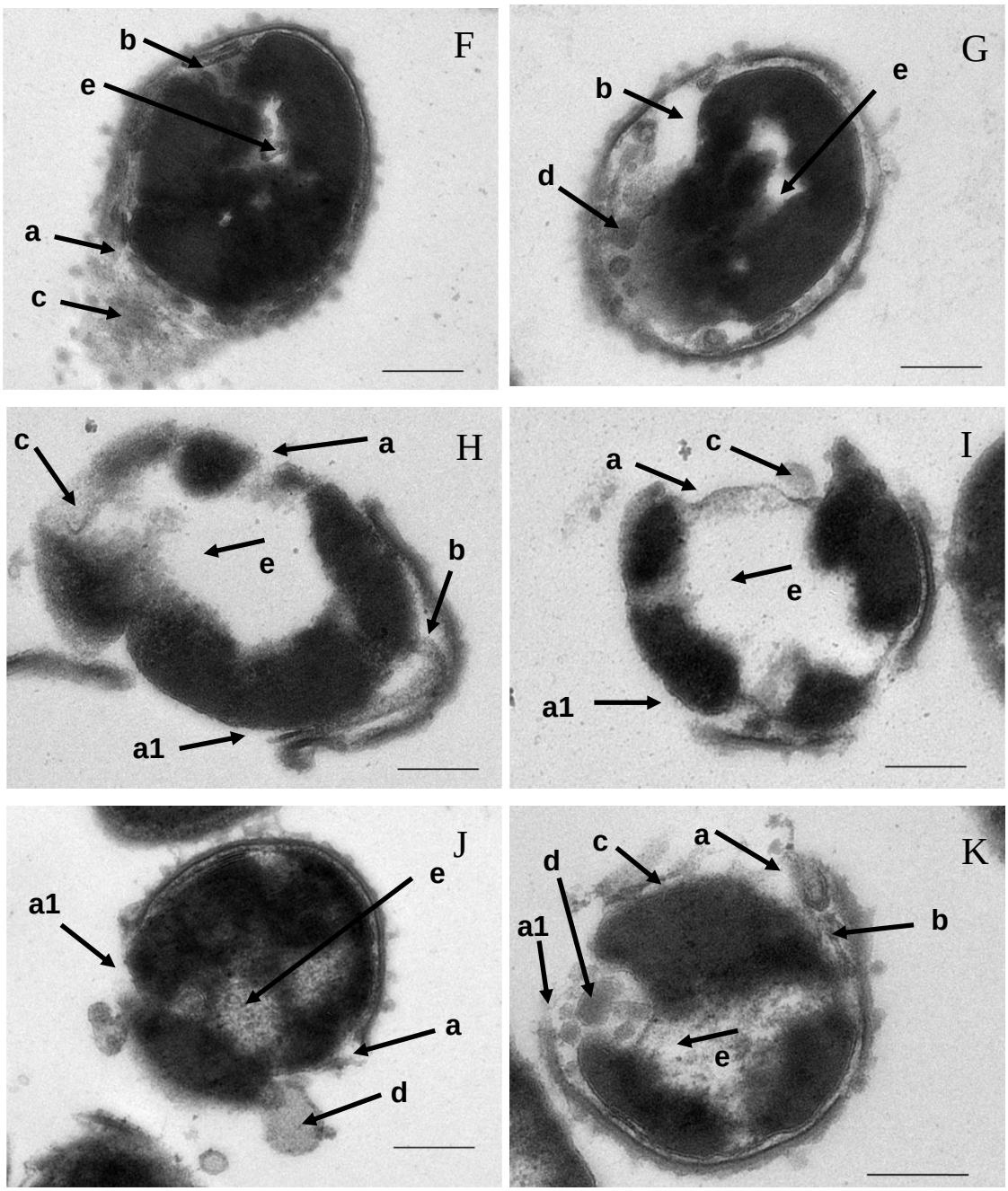


Figure 3.7 (continued): TEM images of PRSP treated with DM1 – DM5. (A) The untreated control showed the normal coccoidal shape morphology of *S. pneumoniae* while severe cell morphological changes following treatment with (B & C) DM1, (D & E) DM2, (F & G) DM3, (H & I) DM4, and (J & K) DM5-treated pneumococcal cells were evident: (a) cell wall/membrane breakages, (a1) loss of cell wall, (b) large cavity between cell wall and plasma membrane, (c) leakage of intracellular contents, (d) breakdown of cytoplasm into inclusion bodies, (e) loss of cytoplasm, and (f) filamentation of intracellular contents. Bar indicates 200 nm.

3.2.2. *In vitro* cell toxicity of peptides on human cells

From the hemolytic and cell cytotoxicity assays, the natural AMP 1VM4, the designed DAMP6 and DP6 peptides showed no hemolytic activity (HC_{10} and HC_{50} > 250 µg/ml) (Table 3.13) and no cytotoxicity on both NL20 (Table 3.14) and A549 (Table 3.15) cell lines at IC_{50} of > 250 µg/ml. 1T51, the template used to design DP5 peptides, displayed considerable cell toxicities with HC_{10} at 55.7 µg/ml and possessed higher selectivity against A549 cell line as compared to NL20 cell line. DP5 peptides showed no cell toxicity in both hemolytic and cell cytotoxicity studies. No hemolytic or cytotoxicity was detected for penicillin in the range of concentrations tested.

As for DAMP7 (Table, 3.13, highlighted in yellow), the hemolytic activity level was higher than the template 1HR1 whereby the HC_{10} and HC_{50} of DAMP7 were approximately two-fold lower at 20.6 µg/ml and 93.0 µg/ml as compared to 1HR1 at 42.3 µg/ml and > 250 µg/ml, respectively. However, DAMP7-treated NL20 (Table 3.14, highlighted in yellow) and A549 cells (Table 3.15, highlighted in yellow) had higher percentages of cell viability as compared to 1HR1. The I_{max} of DAMP7 was approximately 10% higher than 1HR1 on NL20 cell line. In contrast, the differences in IC_{50} levels were less pronounced over prolonged treatment with DAMP7 and 1HR1 on the A549 cell line. This is in parallel with the I_{max} values as the difference in cell viability reduced from 10.4% at 24 hrs to 0.2% at 72 hrs. When compared among the two cell lines, cytotoxicity of DAMP7 on NL20 cell line (IC_{50} = 202 - > 250 µg/ml) was found to be lower than on the A549 cell line (IC_{50} = 82 – 102 µg/ml) by approximately 2 – 2.5 fold for all three time points investigated.

Three of the DP7s showed only minimal level of hemolytic (HC_{10} and HC_{50} >250 µg/ml) and cell cytotoxicities (IC_{50} > 248.3 µg/ml). The hybrid peptides DM1, DM4, and DM5 displayed no hemolytic activity (HC_{10} > 250 µg/ml, H_{max} <

1.0%). DM1 was the least cytotoxic peptide among the hybrid series, which showed IC_{50} of > 220 µg/ml against NL20 cell line and ≥195.0 µg/ml against A549 cell line. For DM4 and DM5, the overall cell cytotoxicity against NL20 was lower than A549 cell line although no major difference in cell viability was observed at the I_{max} level (except for DM4-treated A549 cells at 72 hrs). For DM4, the IC_{50} of NL20 cells were 160.2 µg/ml, 167.3 µg/ml, and 205.0 µg/ml while the IC_{50} of A549 cells were 93.3 µg/ml, 103.0 µg/ml, and 107.5 µg/ml for 24 hrs, 48 hrs, and 72 hrs, respectively. The differences in IC_{50} for DM4-treated NL20 cells were 66.9 µg/ml, 64.3 µg/ml, and 97.5 µg/ml over DM4-treated A549 cells. For DM5, the IC_{50} of NL20 cells were 179.2 µg/ml, 188.2 µg/ml, and 191.7 µg/ml while the IC_{50} of A549 cells were 97.0 µg/ml, 106.3 µg/ml, and 101.7 µg/ml for 24 hrs, 48 hrs, and 72 hrs, respectively. The differences in IC_{50} for DM5-treated NL20 cells were 82.2 µg/ml, 81.9 µg/ml, and 90.0 µg/ml over than DM5-treated A549 cells.

Although DM3 was the most hemolytic peptide among the hybrid series (Table, 3.13, highlighted in blue), the hemolytic levels were lower than DAMP7 as is seen in the HC_{50} and HC_{10} of DM3 (> 250 µg/ml and 52.7 µg/ml, respectively). In contrast, the IC_{50} levels of DM3 (24 hrs = 68.5 µg/ml; 48 hrs = 91.5 µg/ml; 72 hrs = 112.2 µg/ml) were lower than DAMP7 (24 hrs = > 250 µg/ml; 48 hrs = 213.7 µg/ml; 72 hrs = 202.7 µg/ml) on NL20 cell line (Table 3.14, highlighted in yellow). Moreover, the I_{max} levels of DM3 (13.3%, 24.0%, and 12.4%) were lower than DAMP7 (56.4%, 42.5%, and 38.5%) at the respective time points. For the A549 cell line, the cytotoxicity of DM3 and DAMP7 was comparable at 48 hrs and 72 hrs posttreatment except that for 24 hrs posttreatment (IC_{50} DM3 = 56.0 µg/ml, DAMP7 = 102.5 µg/ml) (Table 3.15, highlighted in blue). The cytotoxicity of DM3 was observed to be different with respect to different cell lines at which in general, its cytotoxicity

reduced over prolong incubation time. DM2 which was the second most hemolytic hybrid peptide (HC_{10} = 114.3 µg/ml) showed only minimal level of cytotoxicity on NL20 cell line although the cell viability of A549 cell line over extended treatment duration was found to be decreasing from IC_{50} of > 250 µg/ml, 197.7 µg/ml, and 165.0 µg/ml at 24 hrs, 48 hrs, and 72 hrs, respectively.

Table 3.13: Hemolytic activity of natural AMPs and designed AMPs on human erythrocytes.

Peptide	Hemolytic activity (μg/ml)		
	HC_{10}	HC_{50}	H_{max}
1VM4	>250	>250	1.0±0.2
DAMP6	>250	>250	0.1±0.1
DP61	>250	>250	0.2±0.0
DP62	>250	>250	0.5±0.4
DP63	>250	>250	0.3±0.2
1HR1	42.3±5.0	>250	30.8±3.2
DAMP7	20.6±6.7	93.0±16.5	85.2±5.7
DP71	>250	>250	5.3±2.1
DP72	>250	>250	0.4±0.1
DP73	>250	>250	5.6±0.7
1T51	55.7±4.2	154.3±12.7	84.0±6.6
DP51	>250	>250	0.2±0.1
DP52	>250	>250	0.3±0.1
DP53	>250	>250	0.2±0.1
Hybrid series			
DM1	>250	>250	0.5±0.1
DM2	114.3±26.1	>250	13.9±2.0
DM3	52.7±5.5	>250	39.0±2.5
DM4	>250	>250	0.6±0.1
DM5	>250	>250	0.9±0.4
PEN	>4	>4	0.6±0.3

HC_{10} and HC_{50} were defined as the concentrations of peptide causing 10% and 50% hemolysis on human erythrocytes, respectively.
H_{max} was defined as the percentage (%) hemolysis of peptide at the highest concentration tested (all peptides - 250 μg/ml; PEN - 4 μg/ml).
Highlighted in yellow: The hemolytic profile of DAMP7 defined at HC_{10}, HC_{50}, and H_{max}.
Highlighted in blue: The hemolytic profile of DM3 defined at HC_{10}, HC_{50}, and H_{max}.

Table 3.14: Cell cytotoxicity of natural AMPs and designed AMPs on NL20 cell line.

Peptide	NL20 cell line (μg/ml)					
	24 hrs		48 hrs		72 hrs	
	IC_{50}	I_{max}	IC_{50}	I_{max}	IC_{50}	I_{max}
1VM4	>250	90.1±6.3	>250	77.9±10.7	>250	82.9±8.0
DAMP6	>250	104.2±8.9	>250	89.7±5.7	>250	81.1±1.7
DP61	>250	117.0±8.7	>250	95.9±7.0	.>250	96.4±8.6
DP62	>250	92.9±7.4	>250	90.2±5.3	>250	91.0±9.6
DP63	>250	102.5±10.4	>250	96.0±6.3	>250	98.2±5.3
1HR1	213.3±19.4	40.3±8.7	169.5±6.4	32.2±5.7	98.8±20.2	27.2±3.6
DAMP7	>250	56.4±7.9	213.7±22.9	42.5±5.4	202.7±19.8	38.5±3.9
DP71	>250	90.7±3.6	248.3±1.5	50.6±2.6	>250	49.9±3.0
DP72	>250	60.0±10.0	>250	60.3±3.7	>250	56.4±4.0
DP73	>250	86.4±5.2	>250	80.3±6.2	>250	90.2±8.5
1T51	>250	53.7±6.8	237.7±18.8	50.2±6.8	218.0±37.3	43.6±8.4
DP51	>250	109.4±7.0	>250	110.0±8.3	>250	107.7±2.7
DP52	>250	101.4±3.1	>250	109.2±3.5	>250	102.9±5.9
DP53	>250	110.4±2.7	>250	103.0±7.8	>250	110.8±11.8
Hybrid series						
DM1	221.8±7.8	35.3±6.6	235.7±12.9	42.2±8.6	242.2±7.3	45.9±4.3
DM2	>250	64.3±7.0	>250	53.3±9.1	241.5±9.0	46.8±4.0
DM3	68.5±11.0	13.3±2.1	91.5±7.6	24.0±7.6	112.2±8.8	12.4±4.5
DM4	160.2±11.8	6.9±6.8	167.3±5.9	5.9±2.5	205.0±7.0	30.2±2.9
DM5	179.2±3.8	7.3±2.4	188.2±4.9	8.3±4.2	191.7±3.5	9.7±5.4
PEN	>4	105.2±0.2	>4	106.1±6.2	>4	88.3±0.4

IC_{50} was concentration of peptide which reduced the cell viability to 50%.
I_{max} was defined as the percentage (%) cell viability of peptide at the highest concentration tested (all peptides - 250 μg/ml; PEN - 4 μg/ml).
Highlighted in yellow: The cell cytotoxicity of DAMP7 on NL20 cell line defined at IC_{50} and I_{max} for 24, 48, and 72 hrs.
Highlighted in blue: The cell cytotoxicity of DM3 on NL20 cell line defined at IC_{50} and I_{max} for 24, 48, and 72 hrs.

Table 3.15: Cell cytotoxicity of natural AMPs and designed AMPs on A549 cell line.

Peptide	A549 cell line (μg/ml)					
	24 hrs		48 hrs		72 hrs	
	IC_{50}	I_{max}	IC_{50}	I_{max}	IC_{50}	I_{max}
1VM4	≥250	93.6±3.4	≥250	99.5±1.0	≥250	95.8±1.8
DAMP6	≥250	115.0±7.6	≥250	112.0±7.4	≥250	112.2±4.6
DP61	≥250	92.3±2.3	≥250	91.4±8.9	≥250	95.3±7.8
DP62	≥250	94.2±7.6	≥250	92.3±0.4	≥250	89.5±5.3
DP63	≥250	95.6±2.3	≥250	93.8±6.2	≥250	94.4±6.5
1HR1	47.7±14.6	1.8±1.5	62.2±13.2	1.7±1.5	69.2±10.3	3.3±1.9
DAMP7	102.5±8.3	12.2±5.4	84.8±2.5	7.3±3.9	82.0±1.8	3.5±2.3
DP71	>250	59.6±4.9	>250	61.3±8.7	>250	66.0±8.2
DP72	>250	53.5±9.4	>250	66.9±2.3	>250	80.1±1.4
DP73	>250	66.4±7.6	>250	62.9±9.2	>250	61.5±2.4
1T51	50.7±11.0	7.8±2.9	67.8±19.3	8.1±3.5	82.0±8.9	7.3±3.7
DP51	>250	92.3±7.2	>250	94.8±9.3	>250	99.0±1.7
DP52	>250	80.1±7.2	>250	71.3±5.7	>250	83.5±6.6
DP53	>250	89.4±8.9	>250	96.3±6.2	>250	87.7±4.1
Hybrid series						
DM1	215.3±19.7	38.6±6.2	195.0±25.2	39.8±7.7	>250	66.2±7.2
DM2	>250	50.7±7.7	197.7±12.6	24.0±6.3	165±14.2	12.0±4.1
DM3	56.0±9.3	4.2±3.2	80.0±1.7	4.4±2.4	96.2±4.4	3.7±2.1
DM4	93.3±5.1	8.5±3.8	103.0±17.5	8.9±1.4	107.5±16.3	2,9±2.7
DM5	97.0±9.8	9.0±1.0	106.3±15.7	7.8±3.8	101.7±9.5	8.0±3.2
PEN	>4	95.0±0.4	>4	97.3±0.4	>4	91.1±6.0

IC_{50}, concentration of peptide which reduced cell viability to 50%.
I_{max}, percentage (%) cell viability at maximum concentrations. The maximum concentration for all peptides and PEN were 250 μg/ml and 4 μg/ml, respectively.
Highlighted in yellow: The cell cytotoxicity of DAMP7 on A549 cell line defined at IC_{50} and I_{max} for 24, 48, and 72 hrs.
Highlighted in blue: The cell cytotoxicity of DM3 on A549 cell line defined at IC_{50} and I_{max} for 24, 48, and 72 hrs.

3.2.3. *In vivo* toxicity of peptide treatment

The *in vivo* toxicity of the five DMs was assessed following the three dose regimens given to the mice at 2 hrs, 12 hrs, 24 hrs. Each peptide was initially given the highest deliverable dose via subcutaneous (SC, 100 mg/kg), intranasal (IN, 20 mg/kg), and intraperitoneal (IP, 100 mg/kg) routes. The immediate physical and behavioral abnormalities in mice were recorded for the observations of physical stress, shortness of breath, lethargy, and physical inactiveness (Table 3.16). Doses which caused the death of mice or if they appear highly stressed were not selected and lower graded doses were attempted until an appropriate dose which caused mild/no significant stressful responses to the mice. Any occurrence of death of mice was noted. At day 7 posttreatment, the whole blood was collected for whole blood haematogram analysis which included number of red blood cells (RBC), white blood cells (WBC), B neutrophil, S neutrophil, lymphocytes, monocytes, eosinophil, basophil, and thrombocytes, hemoglobin (Hb) concentration, packed cell volume (pCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), and plasma protein concentration. Serum collected was analyzed for serum biochemistry parameters including the concentrations of alanine transaminase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), creatinine, urea, lactate dehydrogenase (LDH), and direct and total bilirubin. The five major organs (spleen, kidney liver, lung, brain) were processed for histological examination.

For mice treated via SC route at the highest deliverable dose of 100 mg/kg, no physical and behavioral abnormality was observed and no death occurred up to day 7 posttreatment. Statistical analysis found no significant difference ($p > 0.05$) on the whole blood haematogram and serum biochemistry parameters of the mice treated with DM3, DM4, and DM5 as compared to the untreated control (Table 3.17). For DM1-treated mice, significantly higher urea levels ($p = 0.033$, 9.45 ± 1.25 mmol/l) as

compared to the untreated control (7.27 ± 0.93 mmol/l) (Table 3.17, highlighted in yellow). DM2-treated mice had lower plasma protein (p = 0.027, 53.67 ± 1.53 g/l) and lower creatinine levels (32.67 ± 0.58 µmol/l) as compared to the untreated control (p = 0.004, 60.33 ± 3.51 g/l and 40.67 ± 0.58 µmol/l, respectively) (Table 3.17, highlighted in green). In all the treatment groups, no significant histological abnormality was observed in the spleen, kidney, liver, lung, and brain of the mice as compared to the untreated control (Figure 3.8).

For mice treated via IN route at the highest deliverable dose of 20 mg/kg, no physical and behavioral abnormality was observed and no death occurred up to day 7 posttreatment. Statistical analysis found no significant difference (p > 0.05) on the whole blood haematogram and serum biochemistry parameters of the mice treated with DM3 and DM4 as compared to the untreated control (Table 3.18). For DM1-treated mice, significantly lower thrombocytes count (p = 0.012, 1006.00 ± 197.73 x 10^9/l) than the untreated control (1464.25 ± 209.39 x10^9 /l) were noted (Table 3.18, highlighted in yellow). DM2 treatment mice had significantly lower thrombocytes count (p < 0.001, 729.50 ± 253.5 x 10^9/l) which was approximately half of the thrombocytes count in the untreated control (Table 3.18, highlighted in blue). DM2-treated mice also had significant elevated ALP level (p = 0.026, 220.00 ± 16.00 U/l) as compared to the untreated group (136.25 ± 10.34 U/l). Mice treated with DM5 were determined with lower creatinine levels (p = 0.028, 22.5 ± 11.73 µmol/l) than the untreated control (45.5 ± 11.24 µmol/l) (Table 3.18, highlighted in green). In all the treatment groups, no significant histological abnormality was observed in the spleen, kidney, liver, lung, and brain of the mice as compared to the untreated control (Figure 3.9).

For mice treated via IP route at the highest deliverable dose of 100 mg/kg, various physical and behavioral abnormalities were observed with all five DMs treatments (Table 3.16). The mice appeared highly stressed, highly lethargic, and became inactive.

Deaths within 24 hrs were noted for DM3 at 60 mg/kg (2/4 mice died), 80 mg/kg (4/4 mice died) and 100 mg/kg (4/4 mice died). Lower graded doses were attempted and mice receiving DM1 (5 mg/kg), DM2 (60 mg/kg), DM3 (40 mg/kg). DM4 (5 mg/kg), and DM5 (20 mg/kg) displayed no signs of high physical stress and no death occurred (Table 3.16, highlighted in yellow). Thus, each DM was tested at the selected dose via IP route. Statistical analysis on the whole blood haematogram and serum biochemistry parameters showed that mice receiving DM1 (5 mg/kg) and DM2 (60 mg/kg) treatments had no significant difference in any of the parameters as compared to the untreated control (Table 3.19). For mice treated with DM3 (Table 3.19, highlighted in yellow) at 40 mg/kg ($p = 0.002$, 52.25 ± 2.63 fl) and DM5 (Table 3.19, highlighted in blue) at 20 mg/kg ($p = 0.007$, 53.00 ± 2.83 fl), significantly lower MCV was detected as compared to the untreated control (57.75 ± 0.96 fl). DM4-treated mice had lower AST level ($p = 0.022$, 50.88 ± 10.05 U/l) as compared to the untreated control (98.05 ± 34.38 U/l) (Table 3.19, highlighted in green). In all the treatment groups, no significant histological abnormality was observed in the spleen, kidney, liver, lung, and brain of the mice as compared to the untreated control (Figure 3.10).

For mice receiving low doses of DM3 at 10 mg/kg and 20 mg/kg, the mice appeared stressed which resolved within 20 mins and remained physically active (Table 3.16, highlighted in blue). No death occurred and no significant difference ($p > 0.05$) in any of the whole blood and serum biochemistry parameters was noted (Table 3.20). In all the treatment groups, no significant histological abnormality was observed in the spleen, kidney, liver, lung, and brain of the mice as compared to the untreated control (Figure 3.11).

The *in vivo* toxicity of four combinations of DM3 and penicillin were tested together via IP route: $DM3_{10} – PEN_{10}$ (10 mg/kg DM3 and 10 mg/kg PEN), $DM3_{10} – PEN_{20}$ (10 mg/kg DM3 and 20 mg/kg PEN), $DM3_{20} – PEN_{10}$ (20 mg/kg DM3 and 10

mg/kg PEN), and $DM3_{20}$ – PEN_{20} (20 mg/kg DM3 and 20 mg/kg PEN). The mice displayed only slightly stressed appearance which resolved within 20 mins and remained physically active (Table 3.21). No death occurred. No significant difference ($p > 0.05$) in any of the whole blood and serum biochemistry parameters was noted except the lower urea level in the $DM3_{20}$ – PEN_{20} group (5.95 ± 1.08 mmol/l) as compared to the untreated control ($p = 0.001$, 8.83 ± 0.98 mmol/l) (Table 3.22, highlighted in yellow). In all the treatment groups, no significant histological abnormality was observed in the spleen, kidney, liver, lung, and brain of the mice as compared to the untreated control (Figure 3.12).

Table 3.16: The physical and behavioral abnormalities observed in mice following administration of the five DMs at the respective doses via different routes.

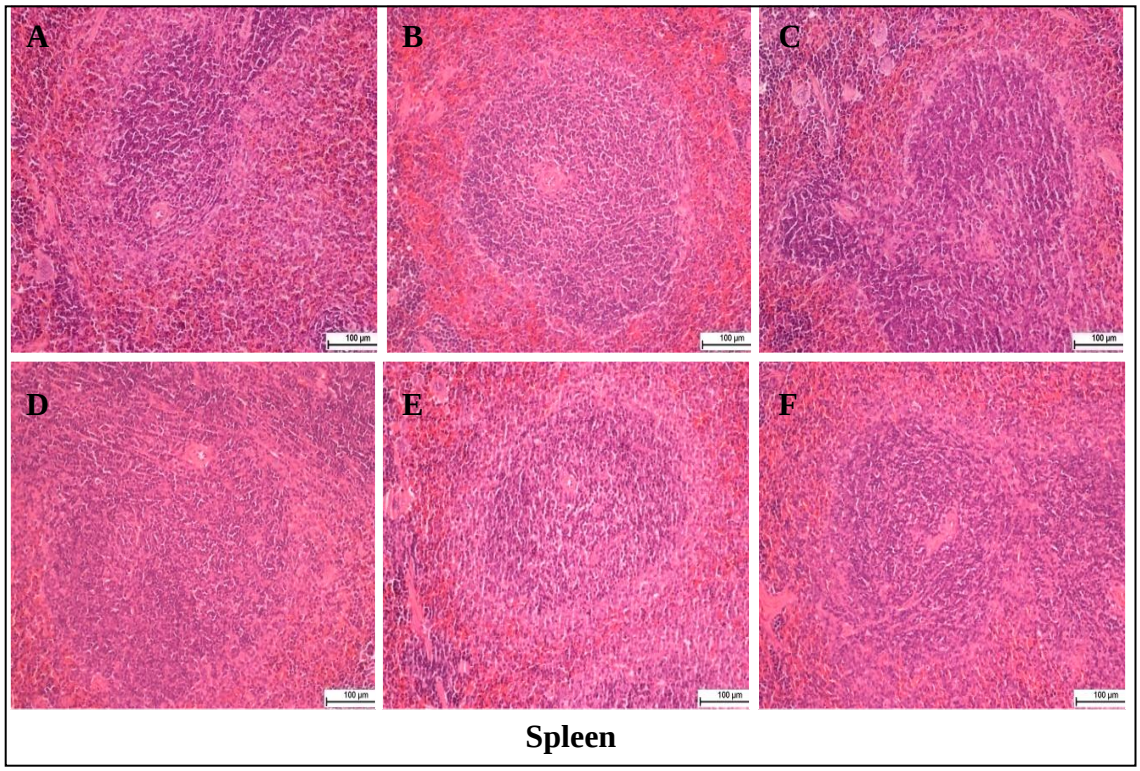
Route	Peptide	Dose (mg/kg)	Observations	No. of dead mice
IP	DM1	100, 80, 60, 40, 20	• Highly stressed and lethargic, shortness of breath, highly inactive.	-
		10	• Moderately stressed and lethargic, shortness of breath, moderately inactive.	-
		5	• Slightly stressed and lethargic (resolved in 30 mins), no apparent shortness of breath, physically active.	-
	DM2	100, 80	• Highly stressed and lethargic, shortness of breath, highly inactive.	-
		60	• Slightly stressed and lethargic (resolved in 30 mins), no apparent shortness of breath, physically active.	-
	DM3	100	• Highly stressed and lethargic, shortness of breath, highly inactive.	4/4
		80	• Highly stressed and lethargic, shortness of breath, highly inactive.	4/4
		60	• Moderately stressed and lethargic, shortness of breath, moderately inactive.	2/4
		40	• Slightly stressed and lethargic (resolved in 30 mins), no apparent shortness of breath, physically active.	-
		20	• Slightly stressed and lethargic (resolved in 20 mins), no apparent shortness of breath, physically active.	-
		10	• Slightly stressed and lethargic (resolved in 20 mins), no apparent shortness of breath, physically active.	-
	DM4	100, 80, 60, 40	• Highly stressed and lethargic, shortness of breath, highly inactive.	-
		20, 10	• Moderately stressed and lethargic, shortness of breath, moderately inactive.	-
		5	• Slightly stressed and lethargic (resolved in 30 mins), no apparent shortness of breath, physically active.	-
	DM5	100, 80, 60	• Highly stressed and lethargic, shortness of breath, highly inactive.	- -
		40	• Moderately stressed and lethargic, shortness of breath, moderately inactive.	-
		20	• Slightly stressed and lethargic (resolved in 30 mins), no apparent shortness of breath, physically active.	-
SC	DM1 DM2 DM3 DM4 DM5	100	• Normal, no signs of stress and lethargy, physically active.	-
IN	DM1 DM2 DM3 DM4 DM5	20	• Normal, no signs of stress and lethargy, physically active.	-

Highlighted in yellow: Highest deliveable dose of each DM given via IP route to the mice.
Highlighted in blue: Lower graded doses of DM3 given via IP route to the mice.
Abbreviations: IP, intraperitoneal; SC, subcutaneous; IN, intranasal.

Table 3.17: Whole blood haematogram and serum biochemistry of mice treated by the DMs via subcutaneous route.

Parameter	SC treatment[a]					
	Untreated (water)	DM1 (100 mg/kg)	DM2 (100 mg/kg)	DM3 (100 mg/kg)	DM4 (100 mg/kg)	DM5 (100 mg/kg)
Whole blood						
Erythrocytes, RBC ($x10^{12}$/l)	8.05±0.11	8.13±0.65	7.39±0.44	8.36±0.60	8.07±0.16	8.22±0.62
Hemoglobin, Hb (g/l)	136.67±1.15	139.75±13.57	130.33±5.13	143.67±5.03	137.67±2.08	142.00±8.98
Packed cell volume, pCV (l/l)	0.46±0.00	0.47±0.04	0.43±0.02	0.49±0.02	0.45±0.00	0.47±0.04
Mean corpuscular volume, MCV (fl)	57.33±0.58	57.75±0.96	58.33±0.58	58.33±2.52	55.67±1.53	57.25±1.50
Mean corpuscular hemoglobin concentration, MCHC (g/l)	297.33±2.31	297.25±3.40	303.00±3.61	295.33±3.06	305.67±4.73	302.25±4.65
While blood cells, WBC ($x10^9$/l)	4.05±1.58	4.21±0.81	2.92±0.42	4.63±1.21	3.31±0.54	3.85±1.13
B neutrophil ($x10^9$/l)	0.06±0.04	0.09±0.05	0.04±0.02	0.14±0.03	0.06±0.03	0.10±0.06
S neutrophil ($x10^9$/l)	0.91±0.37	0.85±0.18	0.90±0.07	1.01±0.18	0.85±0.13	1.10±0.45
Lymphocytes ($x10^9$/l)	2.66±1.13	2.94±0.69	1.68±0.36	3.10±1.18	2.06±0.32	2.26±0.57
Monocytes ($x10^9$/l)	0.31±0.14	0.25±0.04	0.19±0.05	0.31±0.15	0.24±0.06	0.26±0.14
Eosinophil ($x10^9$/l)	0.11±0.10	0.08±0.05	0.10±0.01	0.07±0.03	0.10±0.04	0.14±0.11
Basophil ($x10^9$/l)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Thrombocytes ($x10^9$/l)	1181.33±95.09	1104.50±190.14	1082.00±66.19	1195.00±41.24	1125.33±172.00	1162.00±87.91
Plasma protein (g/l)	60.33±3.51	58.75±2.99	53.67±1.53	61.00±3.61	61.33±1.15	59.75±1.71
Serum biochemistry						
Alanine transaminase, ALT (U/l)	35.50±4.19	29.25±2.44	28.83±3.09	32.20±2.72	38.20±8.00	31.50±4.15
Alkaline phosphatase, ALP (U/l)	142.00±9.54	125.00±29.98	123.33±25.03	127.33±22.55	142.00±28.21	126.50±12.92
Aspartate aminotransferase, AST (U/l)	121.03±4.86	135.00±21.51	137.67±19.00	107.70±4.07	125.10±35.88	91.83±38.33
Creatinine (µmol/l)	40.67±0.58	36.75±3.77	32.67±0.58	37.33±2.08	36.67±2.52	37.75±2.22
Urea (mmol/l)	7.27±0.93	9.45±1.25	8.60±0.95	7.50±0.40	8.10±0.44	8.08±1.06
Lactate dehydrogenase, LDH (U/l)	1451.33±192.20	1286.25±377.56	1664.33±222.07	1214.67±243.24	1046.67±281.95	1044.25±640.49
Direct bilirubin (µmol/l)	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00
Total bilirubin (µmol/l)	2.30±0.46	1.88±0.43	1.83±0.31	2.13±1.02	1.87±0.50	2.05±0.50

[a]Given for 3 doses (2 hrs, 12 hrs, and 24 hrs).
Statistical analysis between treatment groups and untreated control was performed using One-way ANOVA with *post hoc* Dunnett-t test.
Mean value (s) showing significant difference ($p \leq 0.05$) as compared to the untreated control was highlighted:
Highlighted in yellow: DM1-treated mice (Urea, $p = 0.033$).
Highlighted in green: DM2-treated mice (Plasma protein, $p = 0.027$; Creatinine, $p = 0.004$).



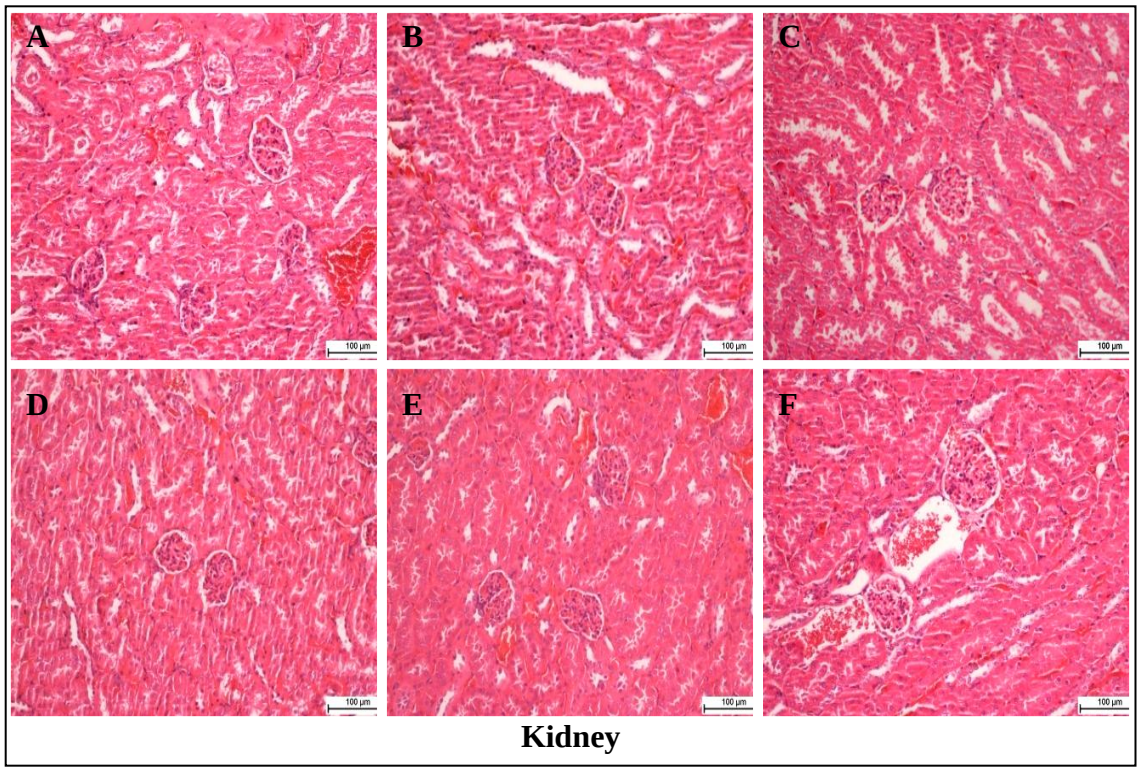
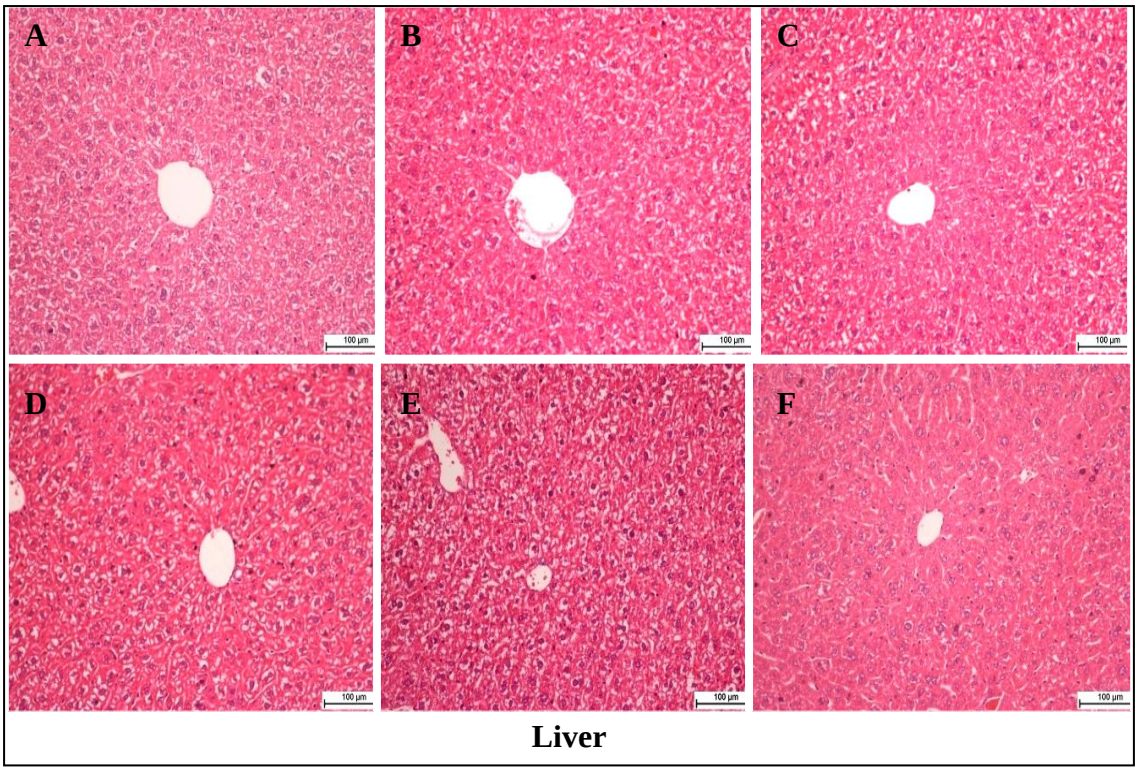


Figure 3.8: Histology of five major organs from mice treated with DM1 – DM5 via SC route at 100 mg/kg for *in vivo* toxicity study.



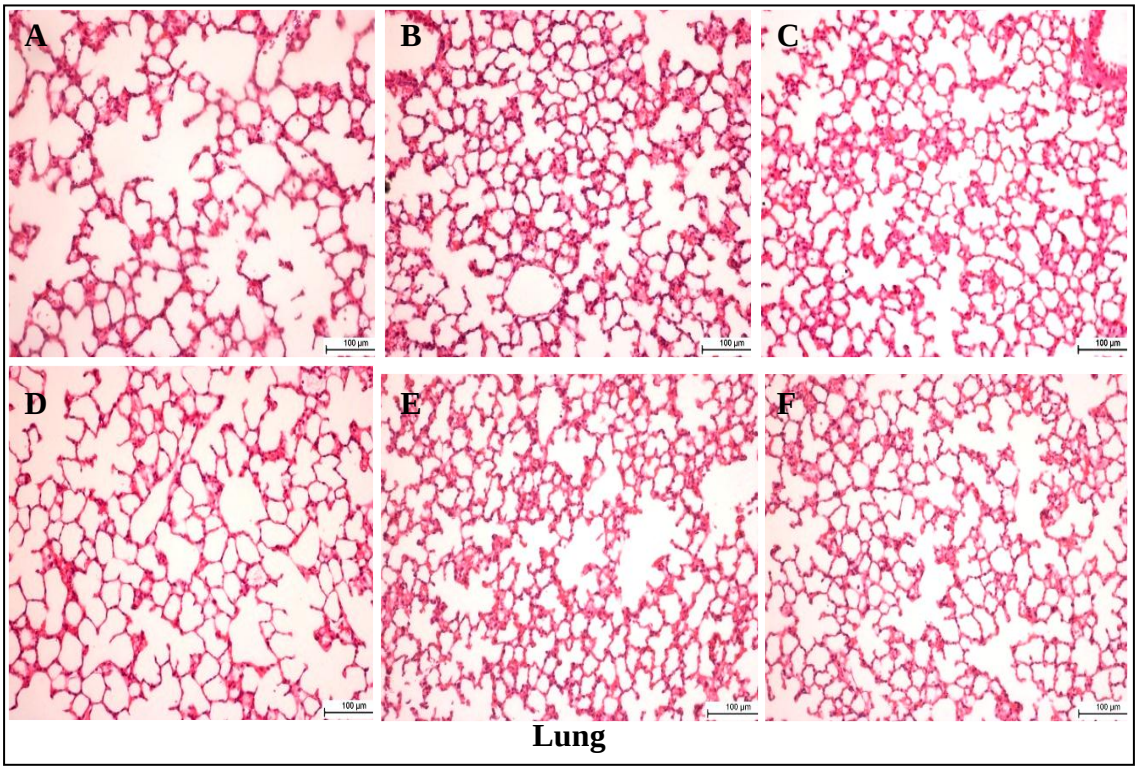


Figure 3.8 (continued): Histology of five major organs from mice treated with DM1 – DM5 via SC route at 100 mg/kg for *in vivo* toxicity study.

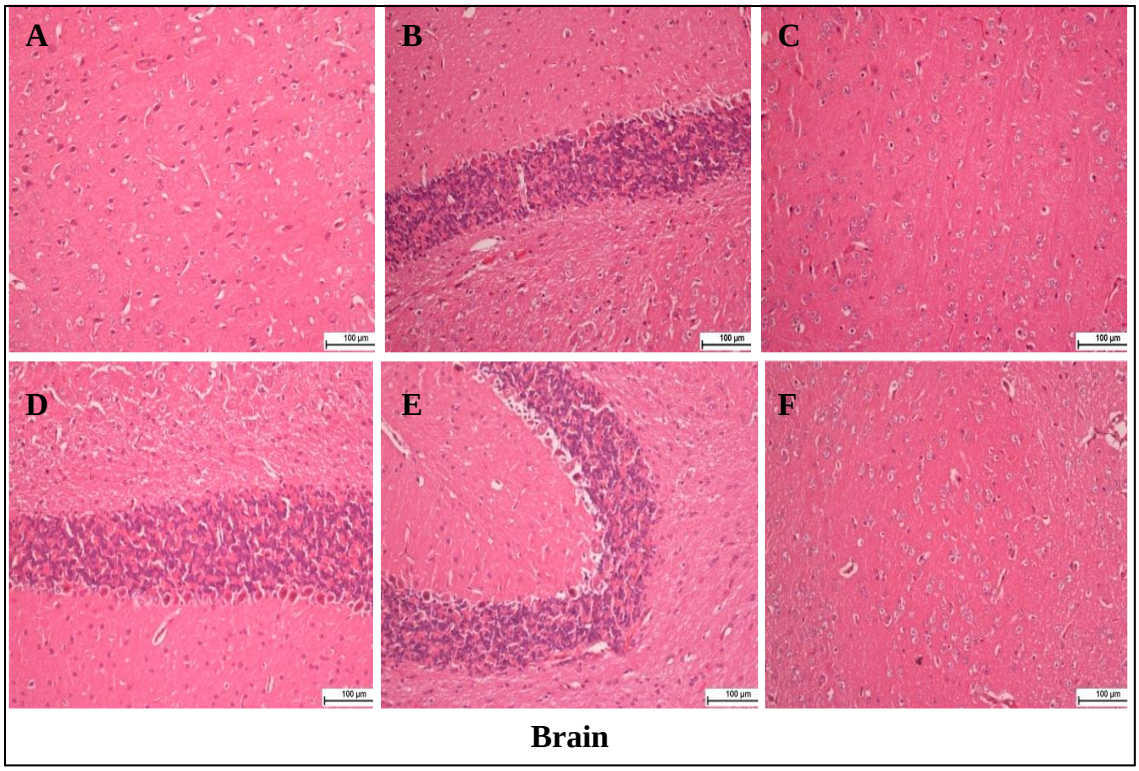
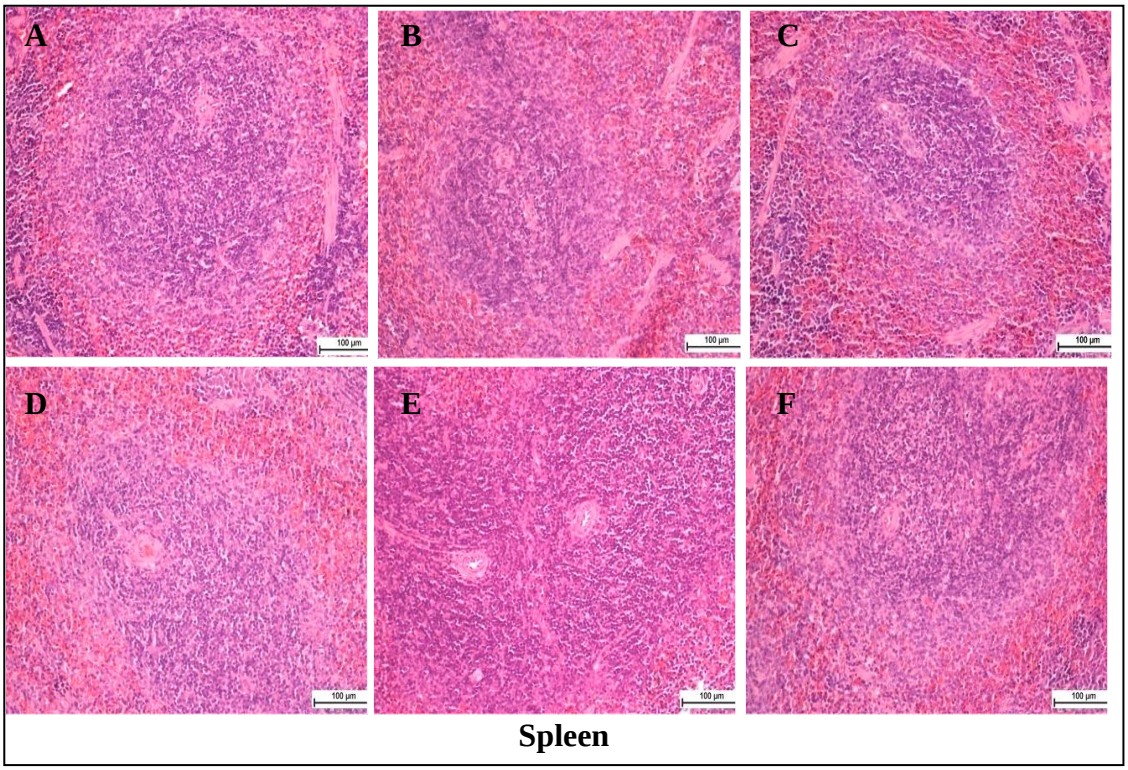


Figure 3.8 (continued): Histology of five major organs from mice treated with DM1 – DM5 via SC route at 100 mg/kg for *in vivo* toxicity study. Spleen, kidney, liver, lung, and brain of untreated control mice (water only) and mice receiving the respective DM for three dose regimens (2 hrs, 12 hrs, 24 hrs) were harvested at day 7 posttreatment and formalin-fixed before processed for histological examination. No significant abnormality was observed in the tissue sections between the (A) untreated control mice and (B) DM1, (C) DM2, (D) DM3, (E) DM4, and (F) DM5-treated mice. Magnification at 200X, H & E staining. Bar indicates 100 µm.

Table 3.18: Whole blood haematogram and serum biochemistry of mice treated by the DMs via intranasal route.

Parameter	IN treatment[a]					
	Untreated (water)	DM1 (20 mg/kg)	DM2 (20 mg/kg)	DM3 (20 mg/kg)	DM4 (20 mg/kg)	DM5 (20 mg/kg)
Whole blood						
Erythrocytes, RBC ($x10^{12}$/l)	7.71±0.84	8.86±0.95	7.87±0.19	8.61±0.71	8.71±0.56	7.84±0.38
Hemoglobin, Hb (g/l)	131.00±2.94	147.67±15.95	130.00±4.00	147.25±9.22	145.75±9.54	134.00±5.72
Packed cell volume, pCV (l/l)	0.44±0.01	0.49±0.03	0.43±0.01	0.48±0.03	0.48±0.03	0.45±0.02
Mean corpuscular volume, MCV (fl)	57.00±6.16	55.00±2.65	54.89±0.19	56.25±1.50	55.25±1.71	57.00±0.82
Mean corpuscular hemoglobin concentration, MCHC (g/l)	301.25±2.99	303.00±13.53	302.42±2.50	305.50±7.33	303.75±7.68	299.50±1.91
While blood cells, WBC ($x10^9$/l)	3.08±0.54	2.27±0.40	3.43±0.54	2.95±0.97	2.21±0.91	2.69±1.37
B neutrophil ($x10^9$/l)	0.09±0.03	0.08±0.03	0.07±0.01	0.10±0.05	0.06±0.02	0.10±0.09
S neutrophil ($x10^9$/l)	1.20±0.79	0.99±0.31	1.37±0.21	0.76±0.19	0.71±0.28	1.12±0.77
Lymphocytes ($x10^9$/l)	1.45±0.32	0.99±0.18	1.64±0.22	1.82±0.66	1.20±0.51	1.25±0.38
Monocytes ($x10^9$/l)	0.24±0.12	0.20±0.07	0.31±0.05	0.25±0.10	0.16±0.05	0.21±0.14
Eosinophil ($x10^9$/l)	0.10±0.06	0.02±0.02	0.04±0.04	0.03±0.03	0.08±0.13	0.02±0.02
Basophil ($x10^9$/l)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Thrombocytes ($x10^9$/l)	1464.25±209.39	1006.00±197.73	729.50±253.50	1590.00±73.21	1470.25±97.66	1205.75±160.80
Plasma protein (g/l)	59.00±3.46	60.00±2.00	52.50±0.50	56.50±3.42	57.25±5.50	53.50±3.32
Serum biochemistry						
Alanine transaminase, ALT (U/l)	42.28±17.29	41.97±11.63	37.20±3.30	35.23±17.39	28.95±14.40	29.45±7.93
Alkaline phosphatase, ALP (U/l)	136.25±10.34	78.00±30.51	220.00±16.00	112.00±42.41	133.75±45.12	108.75±42.53
Aspartate aminotransferase, AST (U/l)	116.43±29.19	108.33±31.27	128.30±18.80	130.03±54.28	96.88±31.79	104.23±26.59
Creatinine (µmol/l)	45.50±11.24	26.33±11.02	36.00±2.00	28.75±10.34	31.25±11.59	22.50±11.73
Urea (mmol/l)	9.78±0.98	6.43±2.77	8.35±1.45	5.93±2.93	8.43±4.64	6.38±2.30
Lactate dehydrogenase, LDH (U/l)	717.00±125.77	887.67±821.34	678.00±67.00	761.25±259.18	609.00±259.29	547.50±175.16
Direct bilirubin (µmol/l)	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00
Total bilirubin (µmol/l)	2.28±1.05	1.83±0.61	2.75±0.15	2.45±0.51	1.88±0.73	1.53±0.59

[a]Given for 3 doses (2 hrs, 12 hrs, and 24 hrs).
Statistical analysis between treatment groups and untreated control was performed using One-way ANOVA with *post hoc* Dunnett-t test.
Mean value (s) showing significant difference ($p \leq 0.05$) as compared to the untreated control was highlighted:
Highlighted in yellow: DM1-treated mice (thrombocytes, $p = 0.012$).
Highlighted in blue: DM2-treated mice (thrombocytes, $p = < 0.001$; ALP, $p = 0.026$).
Highlighted in green: DM5-treated mice (creatinine, $p = 0.028$).



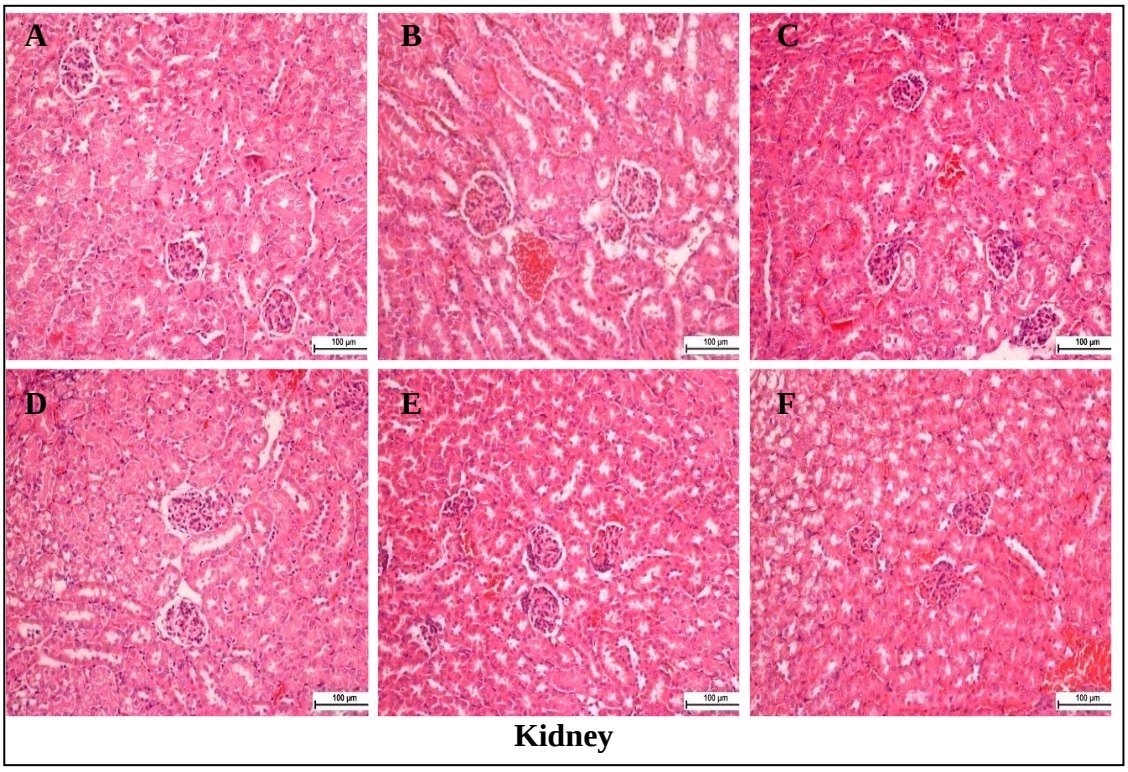
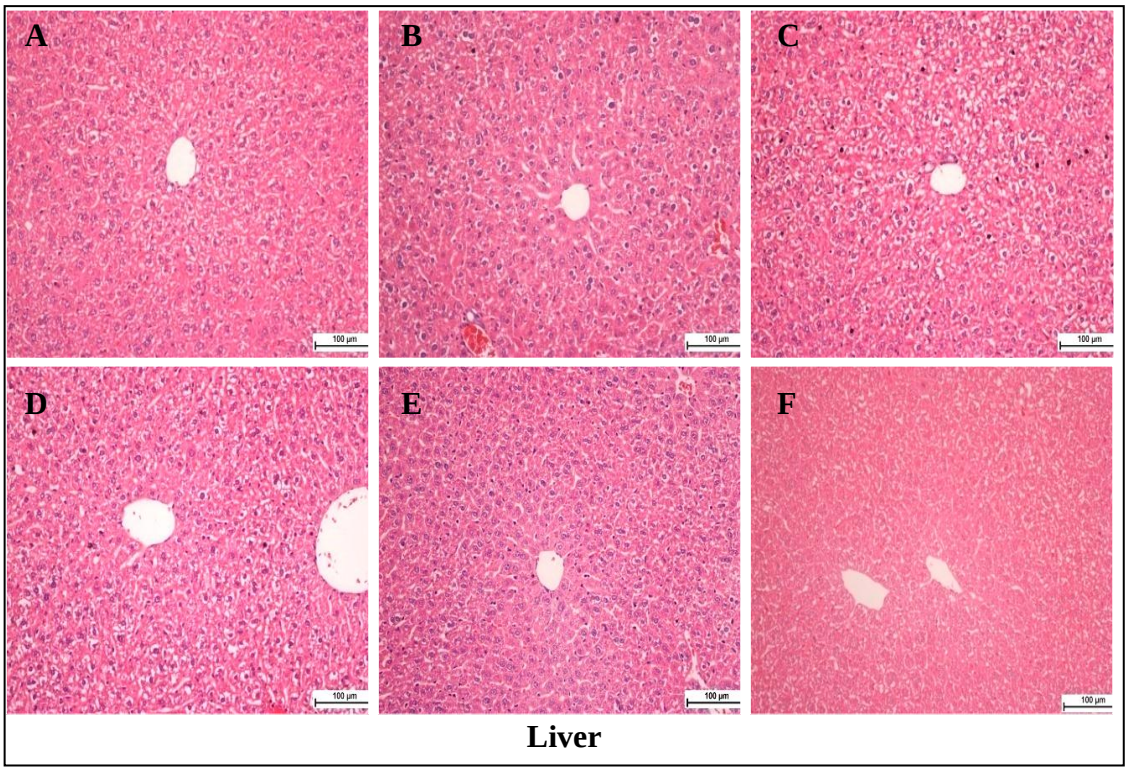


Figure 3.9: Histology of five major organs from mice treated with DM1 – DM5 via IN route at 20 mg/kg for *in vivo* toxicity study.



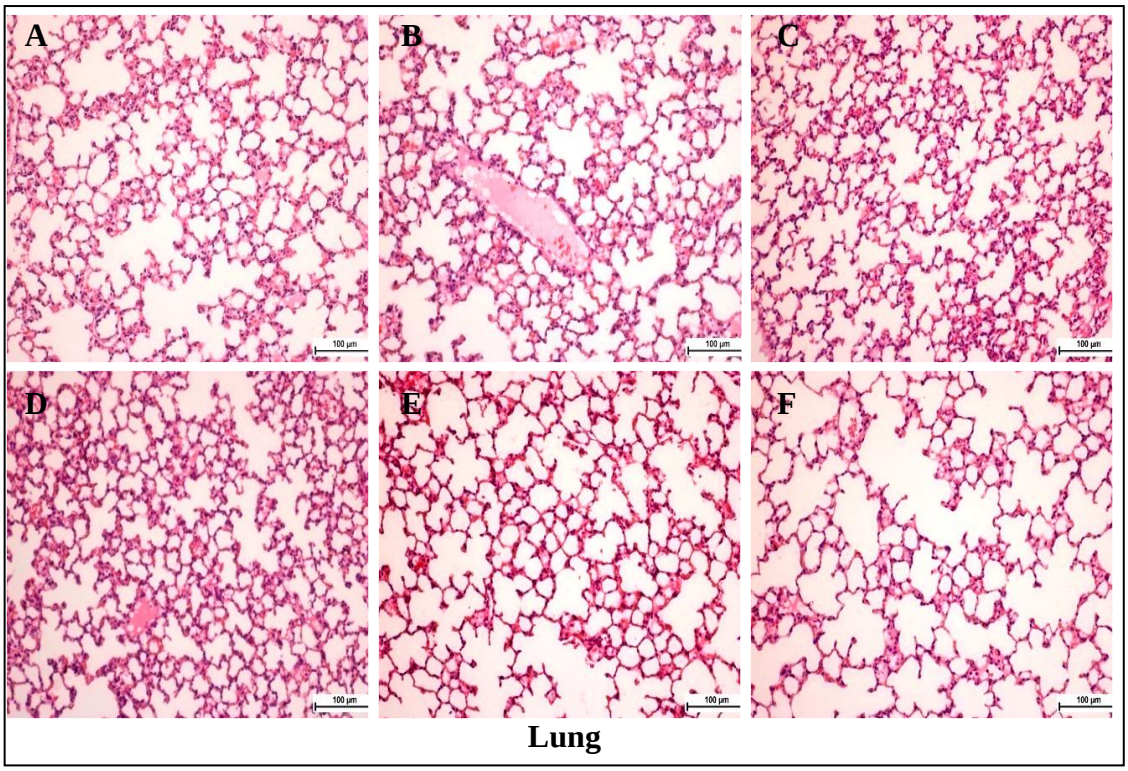


Figure 3.9 (continued): Histology of five major organs from mice treated with DM1 – DM5 via IN route at 20 mg/kg for *in vivo* toxicity study.

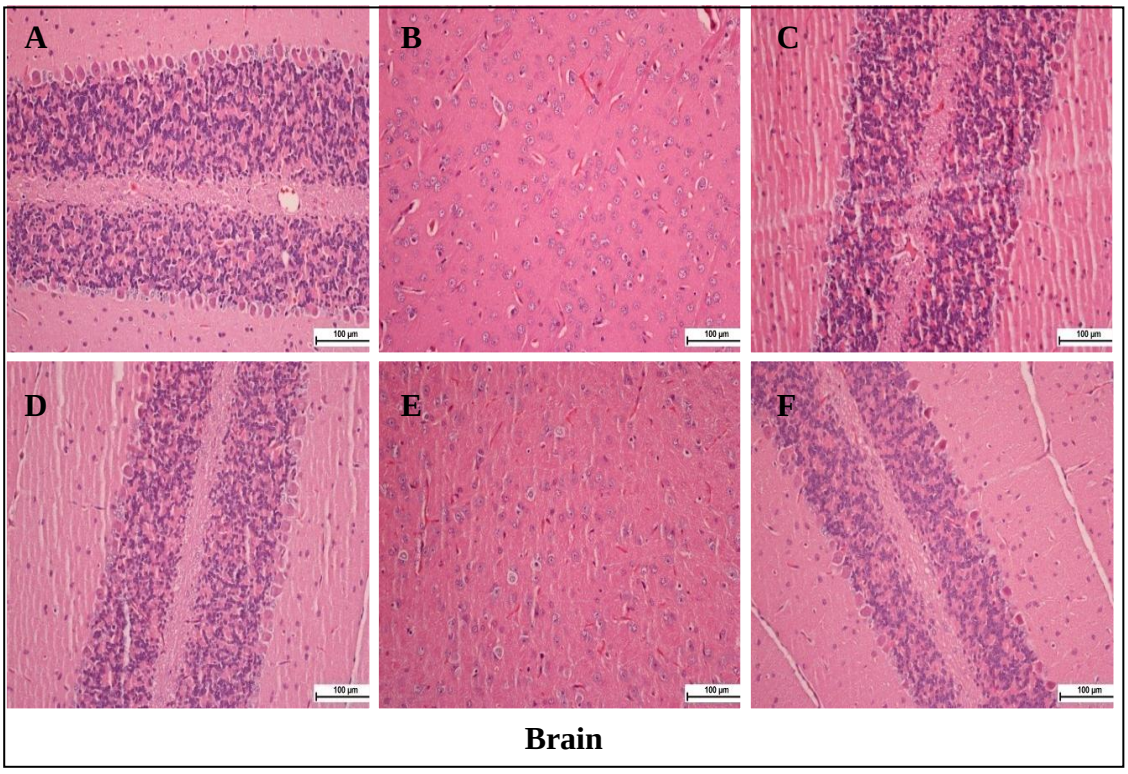
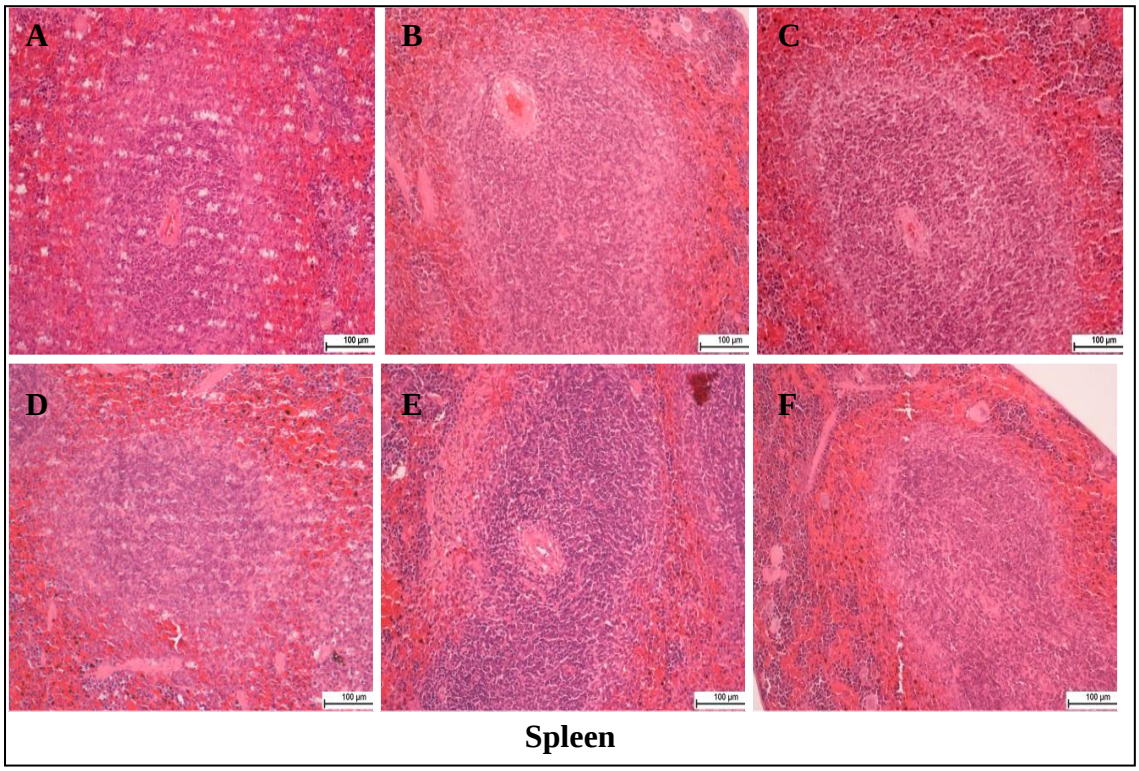


Figure 3.9 (continued): Histology of five major organs from mice treated with DM1 – DM5 via IN route at 20 mg/kg for *in vivo* toxicity study. Spleen, kidney, liver, lung, and brain of untreated control mice (water only) and mice receiving the respective DM for three dose regimens (2 hrs, 12 hrs, 24 hrs) were harvested at day 7 posttreatment and formalin-fixed before processed for histological examination. No significant abnormality was observed in the tissue sections between the (A) untreated control mice and (B) DM1, (C) DM2, (D) DM3, (E) DM4, and (F) DM5-treated mice. Magnification at 200X, H & E staining. Bar indicates 100 µm.

Table 3.19: Whole blood haematogram and serum biochemistry of mice treated by the DMs via intraperitoneal route.

Parameter	IP treatment[a]					
	Untreated (water)	DM1 (5 mg/kg)	DM2 (60 mg/kg)	DM3 (40 mg/kg)	DM4 (5 mg/kg)	DM5 (20 mg/kg)
Whole blood						
Erythrocytes, RBC ($x10^{12}$/l)	7.09±0.31	7.00±0.27	7.55±0.24	7.66±0.60	6.67±0.16	7.93±0.78
Hemoglobin, Hb (g/l)	123.25±5.06	123.00±4.40	131.00±6.22	121.50±10.08	115.25±2.99	127.25±8.96
Packed cell volume, pCV (l/l)	0.41±0.01	0.41±0.02	0.43±0.02	0.40±0.03	0.38±0.01	0.42±0.02
Mean corpuscular volume, MCV (fl)	57.75±0.96	59.00±1.41	56.50±1.29	52.25±2.63	56.50±0.58	53.00±2.83
Mean corpuscular hemoglobin concentration, MCHC (g/l)	300.75±5.68	298.50±6.61	306.50±5.20	303.75±8.10	305.25±2.06	304.75±9.43
While blood cells, WBC ($x10^9$/l)	4.54±1.06	3.23±0.58	3.50±0.61	4.91±0.46	3.42±0.53	4.35±1.07
B neutrophil ($x10^9$/l)	0.15±0.04	0.10±0.04	0.15±0.03	0.15±0.03	0.09±0.02	0.18±0.04
S neutrophil ($x10^9$/l)	1.10±0.34	0.89±0.21	1.11±0.34	1.25±0.21	0.85±0.18	1.26±0.28
Lymphocytes ($x10^9$/l)	2.77±0.66	1.93±0.33	1.87±0.24	2.95±0.26	2.01±0.43	2.30±0.48
Monocytes ($x10^9$/l)	0.30±0.07	0.22±0.08	0.26±0.20	0.33±0.04	0.26±0.06	0.36±0.20
Eosinophil ($x10^9$/l)	0.21±0.09	0.09±0.04	0.11±0.07	0.23±0.13	0.21±0.13	0.26±0.13
Basophil ($x10^9$/l)	0.01±0.03	0.01±0.02	0.01±0.02	0.00±0.00	0.00±0.00	0.01±0.03
Thrombocytes ($x10^9$/l)	856.50±260.67	929.25±108.85	1082.25±96.85	1202.25±248.66	1098.00±102.49	768.00±247.84
Plasma protein (g/l)	48.25±4.35	50.00±2.83	49.00±2.45	54.00±3.27	47.50±1.73	49.75±3.77
Serum biochemistry						
Alanine transaminase, ALT (U/l)	29.15±5.71	26.70±2.42	33.28±11.64	43.90±5.67	22.33±1.87	42.18±11.03
Alkaline phosphatase, ALP (U/l)	124.25±29.66	125.00±14.83	127.25±23.77	121.67±16.17	116.50±20.94	91.25±10.72
Aspartate aminotransferase, AST (U/l)	98.05±34.38	58.00±10.59	77.05±24.78	59.73±13.73	50.88±10.05	75.60±14.97
Creatinine (µmol/l)	39.75±1.71	35.50±5.26	37.00±2.83	46.00±4.58	39.00±1.63	40.75±1.50
Urea (mmol/l)	6.90±1.02	6.95±1.43	7.50±1.02	7.67±0.47	7.80±1.68	5.80±0.53
Lactate dehydrogenase, LDH (U/l)	986.33±256.87	750.25±94.15	775.75±141.00	659.33±117.52	702.75±113.99	728.75±158.71
Direct bilirubin (µmol/l)[b]	0.10±0.00	0.18±0.15	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00
Total bilirubin (µmol/l)	2.15±1.20	1.88±0.45	2.03±0.30	1.93±0.15	2.28±0.51	1.95±0.34

[a]Given for 3 doses (2 hrs, 12 hrs, and 24 hrs).
[b]Value < 0.1 was assumed to be 0.1 to allow statistical analysis.
Statistical analysis between treatment groups and untreated control was performed using One-way ANOVA with *post hoc* Dunnett-t test.
Mean value (s) showing significant difference ($p \leq 0.05$) as compared to the untreated control was highlighted:
Highlighted in yellow: DM3-treated mice (MCV, $p = 0.002$).
Highlighted in blue: DM5-treated mice (MCV, $p = 0.007$).
Highlighted in green: DM4-treated mice (AST, $p = 0.022$).



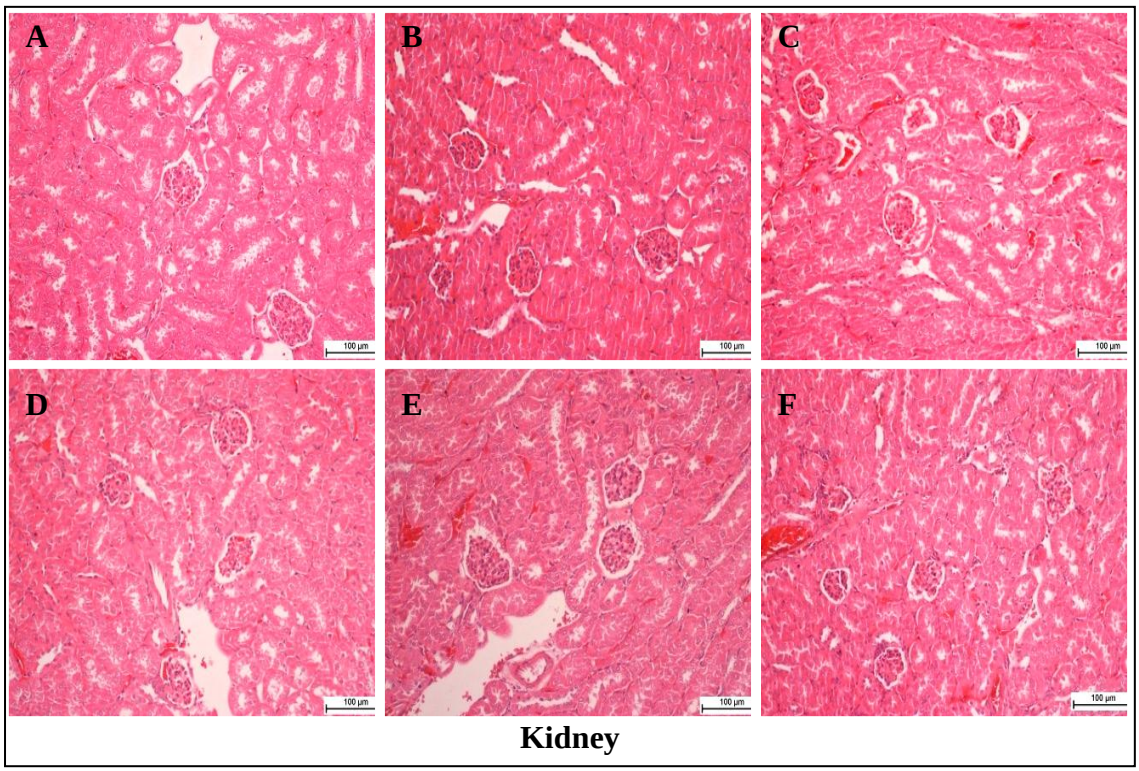
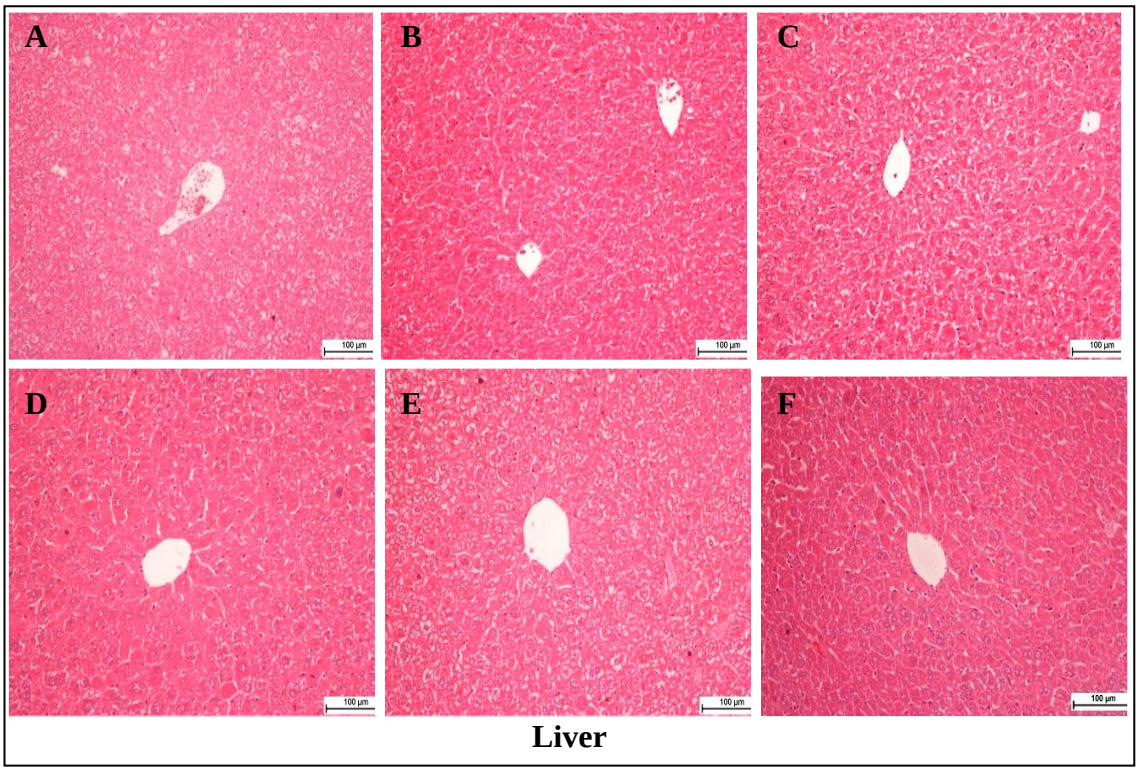


Figure 3.10: Histology of five major organs from mice treated with DM1 – DM5 via IP route at the respective doses for *in vivo* toxicity study.



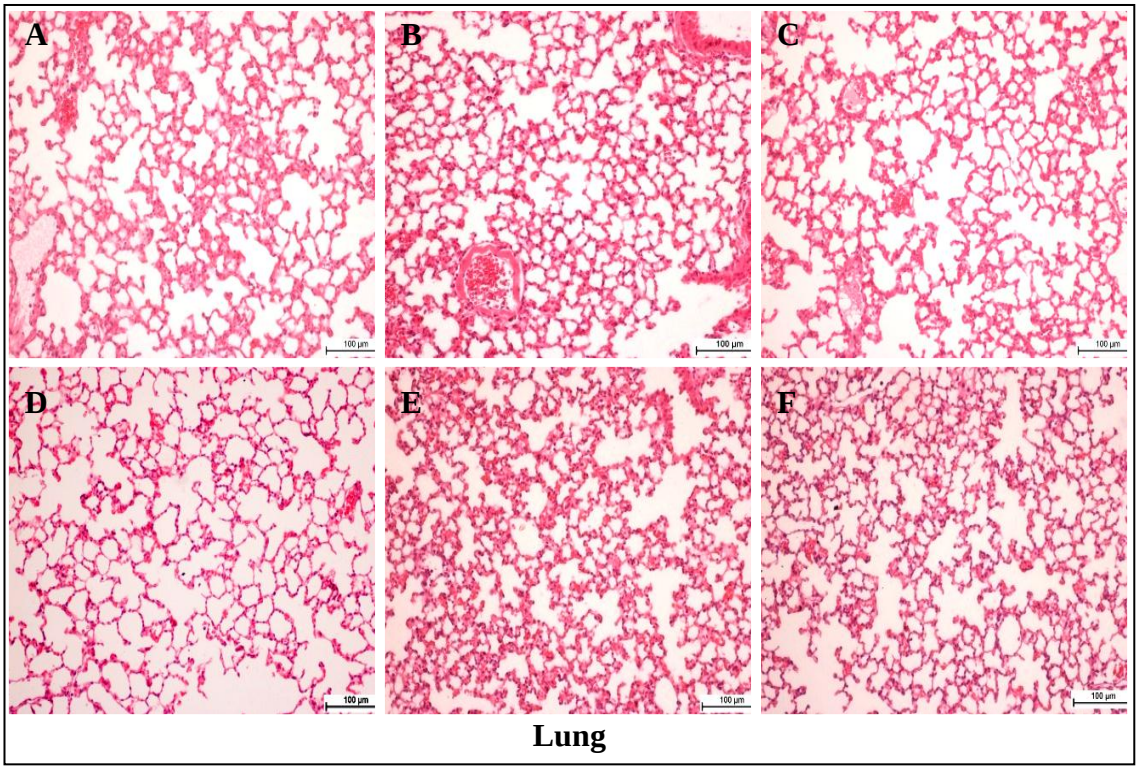


Figure 3.10 (continued): Histology of five major organs from mice treated with DM1 – DM5 via IP route at the respective doses for *in vivo* toxicity study.

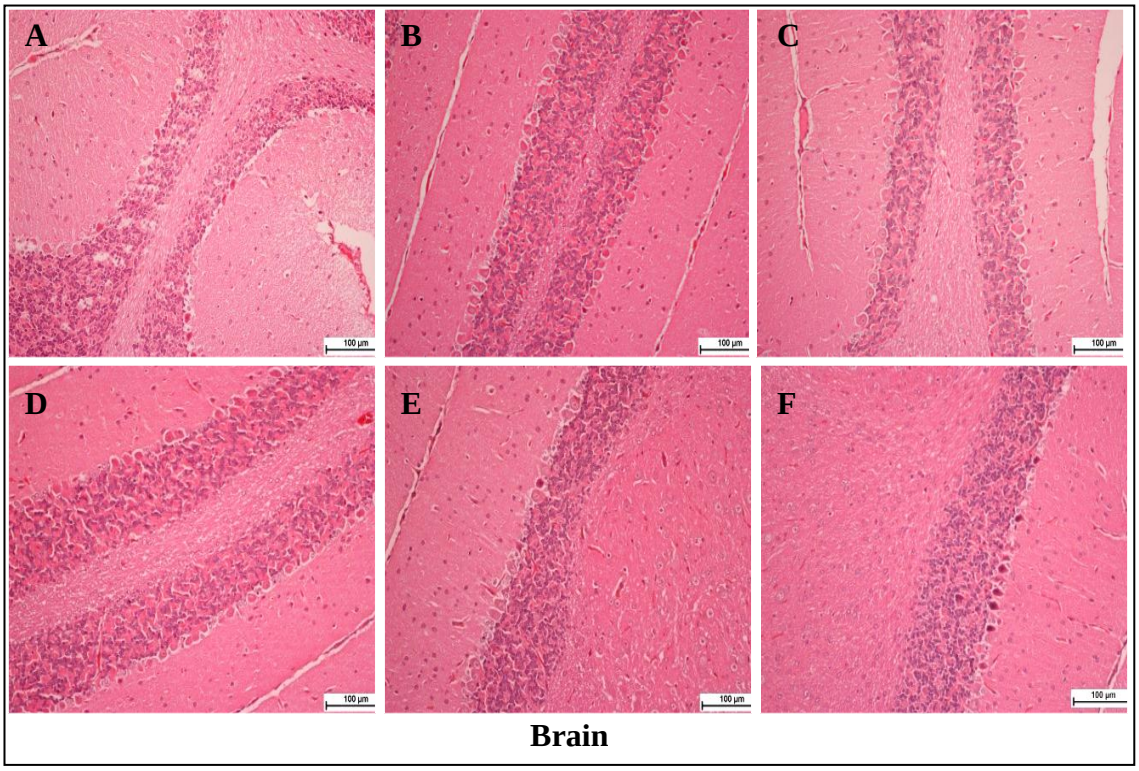


Figure 3.10 (continued): Histology of five major organs from mice treated with DM1 – DM5 via IP route at the respective doses for *in vivo* toxicity study. Spleen, kidney, liver, lung, and brain of untreated control mice (water only) and mice receiving DM1 (5 mg/kg), DM2 (60 mg/kg), DM3 (40 mg/kg), DM4 (5mg/kg), and DM5 (20 mg/kg) for three dose regimens (2 hrs, 12 hrs, 24 hrs) were harvested at day 7 posttreatment and formalin-fixed before processed for histological examination. No significant abnormality was observed in the tissue sections between the (A) untreated control mice and (B) DM1, (C) DM2, (D) DM3, (E) DM4, and (F) DM5-treated mice. Magnification at 200X, H & E staining. Bar indicates 100 µm.

Table 3.20: Whole blood haematogram and serum biochemistry of mice treated with low doses DM3 via intraperitoneal route.

Parameter	IP treatment[a]		
	Untreated (water)	DM3 (10 mg/kg)	DM3 (20 mg/kg)
Whole blood			
Erythrocytes, RBC ($x10^{12}$/l)	6.38±0.47	6.71±0.08	6.90±0.10
Hemoglobin, Hb (g/l)	110.25±8.42	112.50±1.91	116.75±5.56
Packed cell volume, pCV (l/l)	0.38±0.02	0.39±0.01	0.41±0.02
Mean corpuscular volume, MCV (fl)	59.25±1.89	58.50±1.73	58.50±2.65
Mean corpuscular hemoglobin concentration, MCHC (g/l)	292.00±7.96	287.00±3.92	286.50±5.92
While blood cells, WBC ($x10^9$/l)	3.20±0.26	4.07±0.31	4.00±0.53
B neutrophil ($x10^9$/l)	0.11±0.03	0.15±0.03	0.11±0.03
S neutrophil ($x10^9$/l)	0.95±0.24	1.36±0.17	1.12±0.17
Lymphocytes ($x10^9$/l)	1.83±0.36	1.98±0.27	2.28±0.44
Monocytes ($x10^9$/l)	0.20±0.02	0.52±0.13	0.39±0.11
Eosinophil ($x10^9$/l)	0.12±0.03	0.06±0.04	0.11±0.07
Basophil ($x10^9$/l)	0.00±0.00	0.00±0.00	0.00±0.00
Thrombocytes ($x10^9$/l)	931.50±66.29	924.50±103.91	820.75±188.90
Plasma protein (g/l)	42.50±2.52	43.00±0.82	45.50±3.00
Serum biochemistry			
Alanine transaminase, ALT (U/l)	31.58±7.67	36.63±7.45	80.65±51.70
Alkaline phosphatase, ALP (U/l)	194.50±33.93	198.75±78.46	175.00±46.80
Aspartate aminotransferase, AST (U/l)	87.45±14.88	142.43±76.33	127.28±54.67
Creatinine (µmol/l)	28.25±3.30	24.50±3.11	27.50±3.87
Urea (mmol/l)	9.13±0.95	6.70±1.44	7.35±1.70
Lactate dehydrogenase, LDH (U/l)	1767.00±718.75	1184.75±375.55	1243.25±499.73
Direct bilirubin (µmol/l)[b]	0.55±0.57	0.10±0.00	0.10±0.00
Total bilirubin (µmol/l)	1.75±1.28	2.23±1.07	1.53±0.24

[a]Given for 3 doses (2 hrs, 12 hrs, and 24 hrs).
[b]Value < 0.1 was assumed to be 0.1 to allow statistical analysis.
Statistical analysis between treatment groups and untreated control was performed using One-way ANOVA with *post hoc* Dunnett-t test.
Mean value (s) showing significant difference ($p \leq 0.05$) as compared to the untreated control was highlighted (no significant difference was noted).

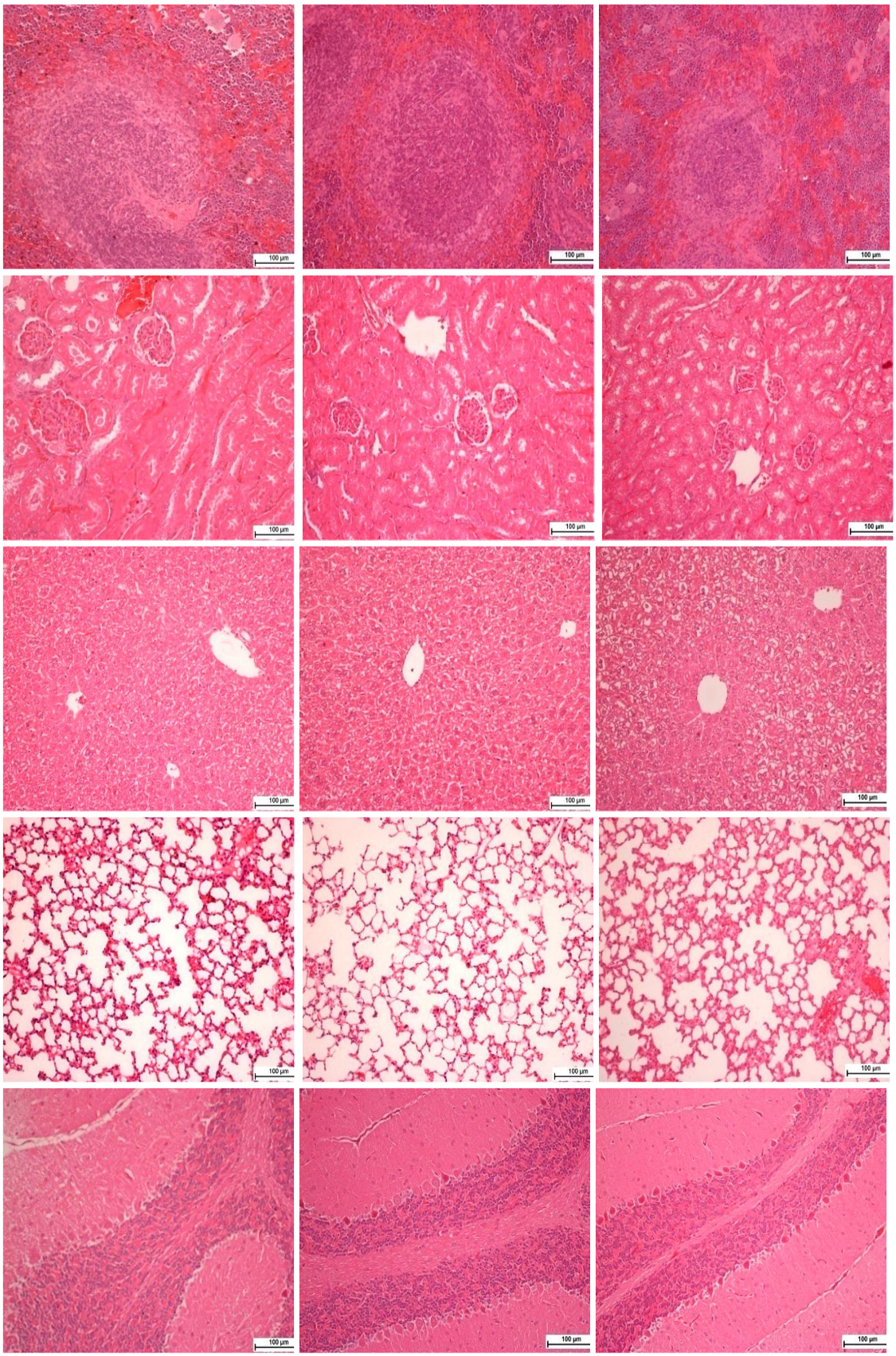


Figure 3.11: Histology of five major organs from mice treated with low doses DM3 via IP route for *in vivo* toxicity study. DM3 at 10 mg/kg (middle column) and 20 mg/kg (right column) were given for three dose regimens (2 hrs, 12 hrs, 24 hrs) and compared to untreated control (left column). Spleen, kidney, liver, lung, and brain (top to bottom) were harvested at day 7 posttreatment and formalin-fixed before processed for histological examination. No significant abnormality was observed in the tissue sections between the untreated control mice and the treated mice. Magnification at 200X, H & E staining. Bar indicates 100 µm.

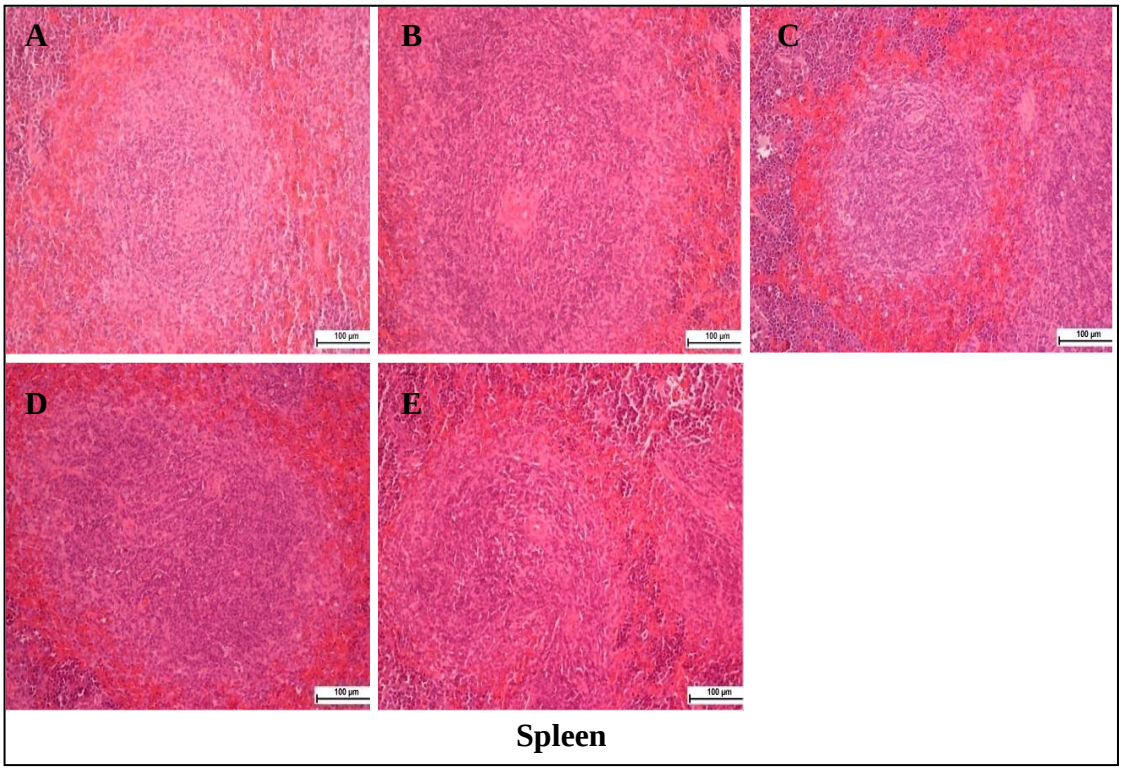
Table 3.21: The physical and behavioral abnormalities observed following administration of the DM3 – PEN combinations via IP route.

Group	Dose (mg/kg)		Observations	No. of dead mice
	DM3	PEN		
$DM3_{10}$-PEN_{10}	10	10	• Slight discomfort (resolved in 20 mins) and then appeared normal, physically active.	-
$DM3_{10}$-PEN_{20}	10	20	• Slight discomfort (resolved in 20 mins) and then appeared normal, physically active.	-
$DM3_{20}$-PEN_{10}	20	10	• Slight discomfort (resolved in 20 mins) and then appeared normal, physically active.	-
$DM3_{20}$-PEN_{20}	20	20	• Slight discomfort (resolved in 20 mins) and then appeared normal, physically active.	-

Table 3.22: Whole blood haematogram and serum biochemistry of mice treated with DM3 – PEN combinations via intraperitoneal route.

Parameter	IP treatment[a]				
	Untreated (water)	$DM3_{10}$ - PEN_{10}	$DM3_{10}$ – PEN_{20}	$DM3_{20}$ - PEN_{10}	$DM3_{20}$ – PEN_{20}
Whole blood					
Erythrocytes, RBC ($x10^{12}$/l)	6.32±0.46	6.14±0.27	6.28±0.30	6.15±0.14	6.18±0.15
Hemoglobin, Hb (g/l)	110.25±8.42	105.75±2.87	111.25±1.89	103.33±4.16	106.75±4.27
Packed cell volume, pCV (l/l)	0.38±0.02	0.36±0.02	0.39±0.01	0.36±0.01	0.37±0.01
Mean corpuscular volume, MCV (fl)	60.25±2.06	59.25±0.96	61.25±2.50	59.00±1.73	60.25±0.96
Mean corpuscular hemoglobin concentration, MCHC (g/l)	290.00±6.06	292.00±8.12	288.75±4.72	284.33±7.09	286.75±8.54
While blood cells, WBC ($x10^9$/l)	2.98±0.50	3.80±1.04	3.58±0.87	3.07±0.76	3.25±0.51
B neutrophil ($x10^9$/l)	0.09±0.04	0.15±0.08	0.11±0.06	0.08±0.02	0.11±0.05
S neutrophil ($x10^9$/l)	0.90±0.23	1.14±0.39	1.00±0.27	0.91±0.27	1.19±0.28
Lymphocytes ($x10^9$/l)	1.75±0.37	2.01±0.46	1.96±0.44	1.60±0.44	1.54±0.43
Monocytes ($x10^9$/l)	0.17±0.04	0.38±0.17	0.35±0.15	0.24±0.11	0.28±0.12
Eosinophil ($x10^9$/l)	0.08±0.04	0.13±0.06	0.16±0.11	0.23±0.22	0.14±0.04
Basophil ($x10^9$/l)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Thrombocytes ($x10^9$/l)	895.00±36.76	879.00±62.25	800.75±55.07	933.00±36.76	818.33±38.08
Plasma protein (g/l)	41.00±1.15	41.25±2.87	44.00±1.63	42.00±3.46	42.00±1.63
Serum biochemistry					
Alanine transaminase, ALT (U/l)	31.50±7.71	23.63±7.59	29.00±6.92	36.00±17.47	34.20±11.88
Alkaline phosphatase, ALP (U/l)	174.50±10.54	183.75±46.36	162.75±5.85	142.33±14.47	137.50±22.35
Aspartate aminotransferase, AST (U/l)	84.15±15.29	81.75±20.16	95.08±36.56	125.37±65.23	95.08±32.83
Creatinine (µmol/l)	25.50±2.52	24.75±4.65	25.00±1.83	23.33±1.15	25.25±1.26
Urea (mmol/l)	8.83±0.98	8.65±1.55	7.50±1.06	6.47±1.04	5.95±1.08
Lactate dehydrogenase, LDH (U/l)	1661.25±819.00	1991.75±785.73	1129.25±84.92	2362.00±432.35	1129.50±771.26
Direct bilirubin (µmol/l)[b]	0.55±0.57	0.23±0.25	0.60±0.53	0.93±1.44	0.68±0.68
Total bilirubin (µmol/l)	1.93±0.67	1.80±0.52	1.73±0.22	2.60±0.82	1.90±0.43

[a]Given for 3 doses (2 hrs, 12 hrs, and 24 hrs).
[b]Value < 0.1 was assumed to be 0.1 to allow statistical analysis.
Statistical analysis between treatment groups and untreated control was performed using One-way ANOVA with *post hoc* Dunnett-t test.
Mean value (s) showing significant difference ($p \leq 0.05$) as compared to the untreated control was highlighted.
Highlighted in yellow: $DM3_{20}$ -PEN_{20} treated mice (Urea, $p = 0.001$).



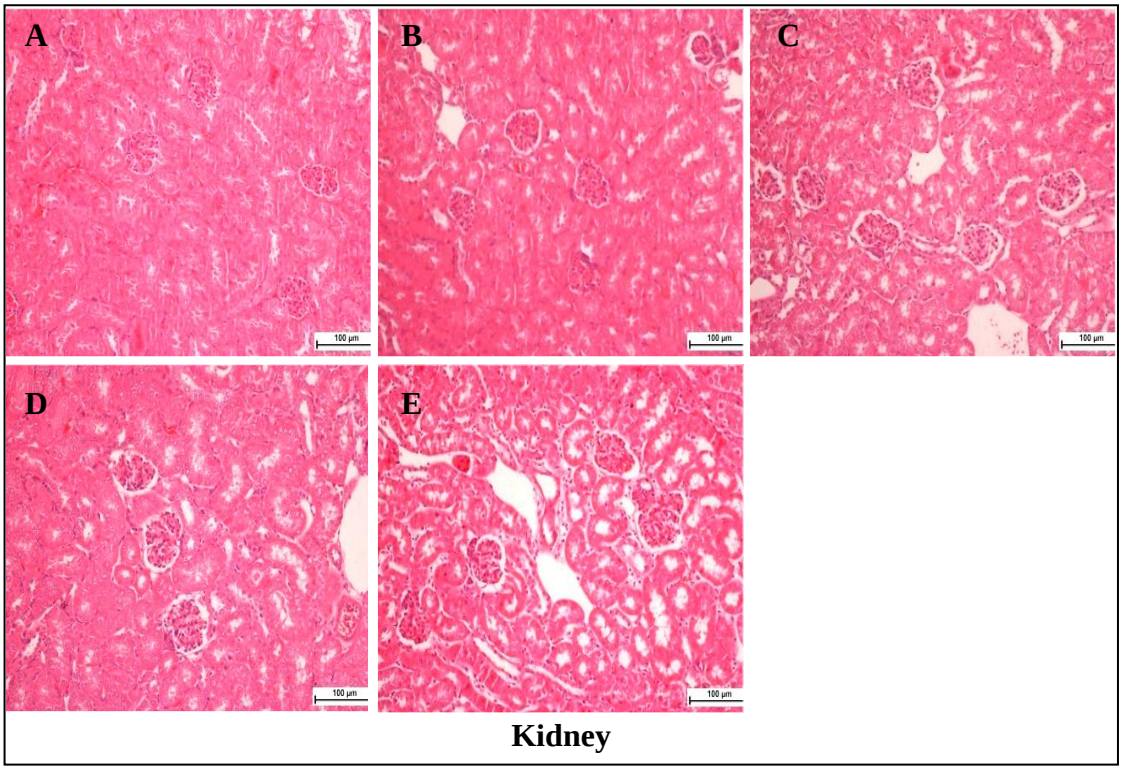
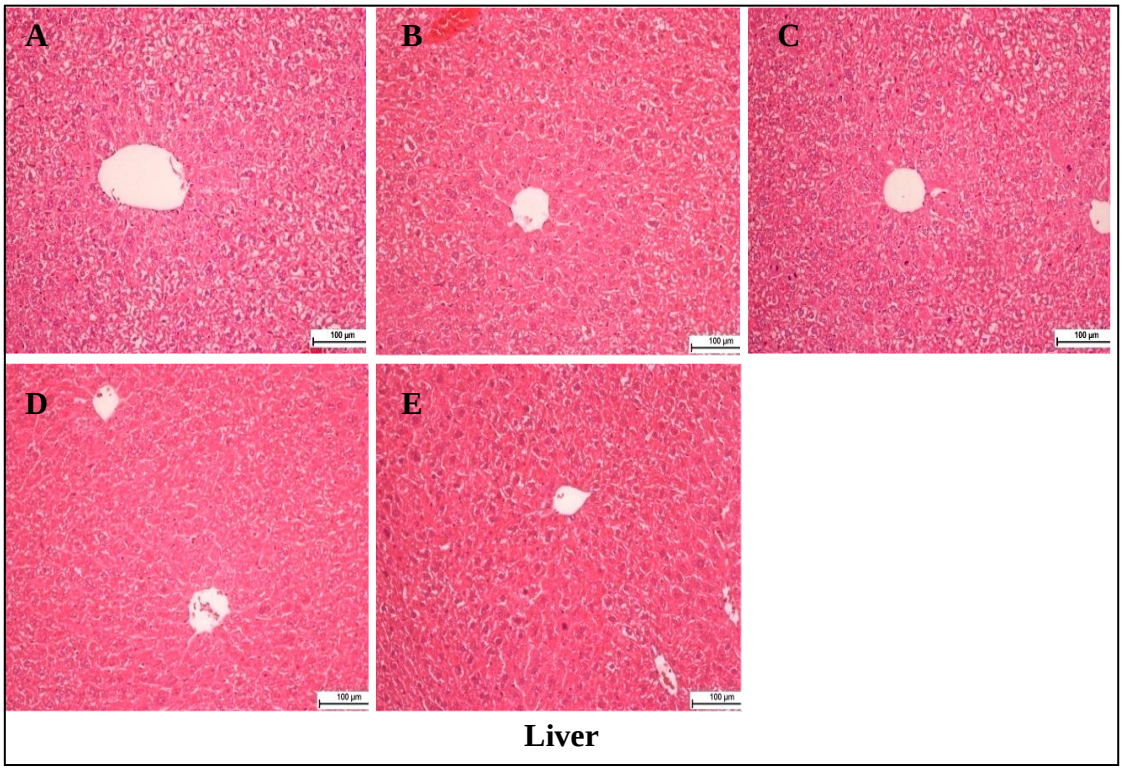


Figure 3.12: Histology of five major organs from mice treated with the combinations of DM3 – PEN via IP route for *in vivo* toxicity study.



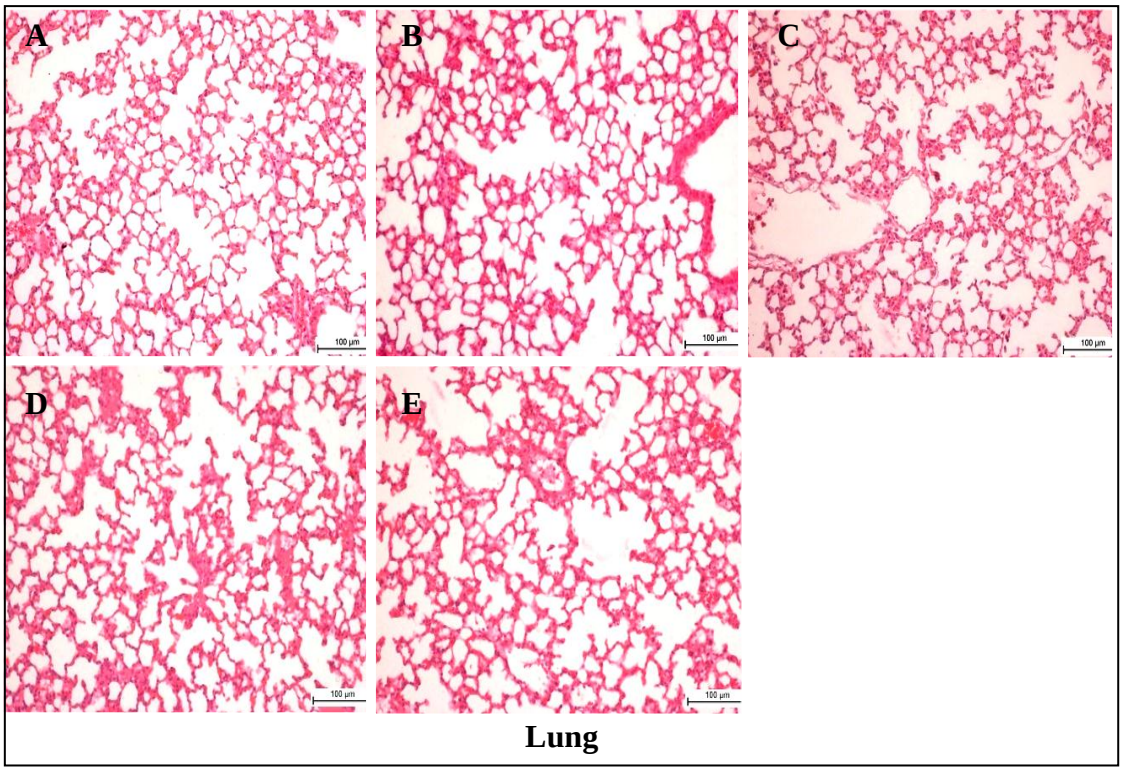


Figure 3.12 (continued): Histology of five major organs from mice treated with the combinations of DM3 – PEN via IP route for *in vivo* toxicity study.

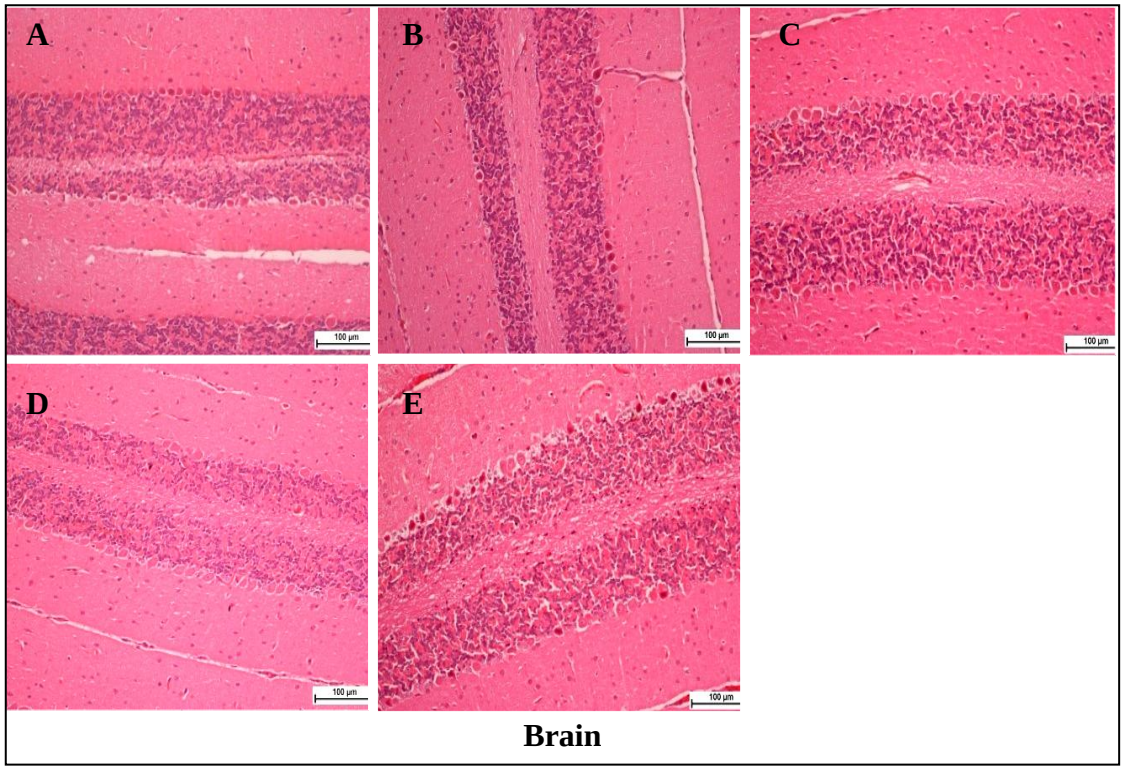


Figure 3.12 (continued): Histology of five major organs from mice treated with the combinations of DM3 – PEN via IP route for *in vivo* toxicity study. Spleen, kidney, liver, lung, and brain of untreated control mice (water only) and mice receiving the respective formulations for three dose regimens (2 hrs, 12 hrs, 24 hrs) were harvested at day 7 posttreatment and formalin-fixed before processed for histological examination. No significant abnormality was observed in the tissue sections between the (A) untreated control mice and (B) $DM3_{10}$-PEN_{10}, (C) $DM3_{10}$-PEN_{20}, (D) $DM3_{20}$-PEN_{10}, and (E) $DM3_{20}$-PEN_{20}-treated mice. Magnification at 200X, H & E staining. Bar indicates 100 µm.

3.2.4. Therapeutic efficacy of peptides in two lethal pneumococcal infection models

Two in-house lethal pneumococcal infection models induced by a penicillin-resistant pneumococcal strain mimicking the two major types of pneumococcal infections in human were developed to assess the therapeutic efficacy of peptides *in vivo*. The lethal pneumococcal systemic infection model was developed by infecting the mice with the selected inoculums of 1.5×10^2 CFU/mouse via IP route to simulate the manifestation of pneumococcal bacteremia while the lethal pneumococcal pneumonia model was developed by infecting the mice with the selected inoculums of 5×10^3 CFU/mouse via intrathoracic route (IT) to simulate the manifestation of pneumococcal pneumonia.

In both models, mice infected with *S. pneumoniae* displayed various symptoms such as hunching of body posture, ruffled fur, lethargy, less responsive to physical sensitization, loss of body temperature, and failed defecations (Figure 3.13A & B). All these infected mice died within two to four days postinfection. Upon necropsy, the lungs of the infected mouse were significantly presented with redness and swelling, depicting severe inflammation. In addition, leakage of body fluid into peritoneal cavity and thoracic cavity, swelling and loss of the arrangement and compaction of digestive tract especially the small and large intestines were observed (Figure 3.13C & D). Cultures of blood and homogenates of five major organs (spleen, kidney, liver, lung, and brain) of the dead/moribund mice following infections via both IP and IT routes detected pneumococci as compared to the uninfected control mice. This indicates the rapid and uncontrolled spread of the inoculated pneumococcus in infecting the organs and establishing severe infection state.

Since all five DMs displayed strong antipneumococcal activities from the *in vitro* studies, the therapeutic efficacy of the peptides were tested in both the infection models.

The treatment regimens for each DM followed the highest deliverable doses that have been determined from the *in vivo* toxicity assessment at 2 hrs, 12 hrs, and 24 hrs postinfection (Table 3.16, highlighted in yellow). For IP route of administration, each DM1, DM2, DM3, DM4, and DM5 were given at 5 mg/kg, 60 mg/kg, 40 mg/kg, 5 mg/kg, and 20 mg/kg, respectively. For SC route of administration, the DMs were given at 100 mg/kg and for IN route of administration, the DMs were given at 20 mg/kg, respectively.

In pneumococcal systemic infection model, preliminary testing using groups of three mice found that mice treated with DM1, DM2, DM3, and DM5 at the respective doses had enhanced survival (Table 3.23, highlighted in yellow). These four peptides were then selected for testing using groups of 10 mice. Results showed that DM3 at 40 mg/kg protected 50% of the lethally infected mice from mortality up to day 7 postinfection with statistically significant survival analysis ($p = 0.004$) (Figure 3.14). Although DM1, DM2, and DM5 appeared to have therapeutic effects in the preliminary testing, subsequent therapeutic efficacy using groups of 10 mice had no mice survived up to day 7 postinfection. Survival analysis up to day 7 postinfection showed that treatment using DM2 ($p = 0.009$) and DM5 ($p = 0.045$) significantly enhanced survival of the systemically infected mice as compared to untreated control. Treatment via SC and IN had no survival at day 7 postinfection and found no significant survival analysis ($p > 0.05$) in the systemic infection model.

Mice receiving penicillin treatments via IP route at 10 mg/kg, 20 mg/kg, 40 mg/kg, and 80 mg/kg were determined to have 20%, 50%, 60%, and 90% survival up to day 7 postinfection and were statistically significant ($p < 0.001$) (Figure 3.15). Moreover, mice receiving the lower graded doses of DM3 at 10 mg/kg and 20 mg/kg had 10% ($p = 0.424$) and 20% ($p = 0.019$) survival rates at day 7 postinfection for which only DM3 at 20 mg/kg conferred statistically significant ($p = 0.019$) survivability to the

mice. All the mice survived up to day 7 were humanely sacrificed and plating of blood and the homogenates of the sample tissues from spleen, kidney, liver, lung, and brain found no growth of *S. pneumoniae*.

In the pneumococcal pneumonia model, treatment via IP, SC, and IN had no survival up to day 7 postinfection (Table 3.23). Thus, these peptides and the respective treatment regimens were not selected for subsequent therapeutic efficacy testing in groups of 10 mice. In all testing, no pneumococcus was detected from blood and the five major organs (spleen, liver, kidney, lung, brain) of the mice that survived and had no presentation of illnesses as compared to the untreated control that grew overcrowded pneumococcal cells from blood agar plating and were severely ill.

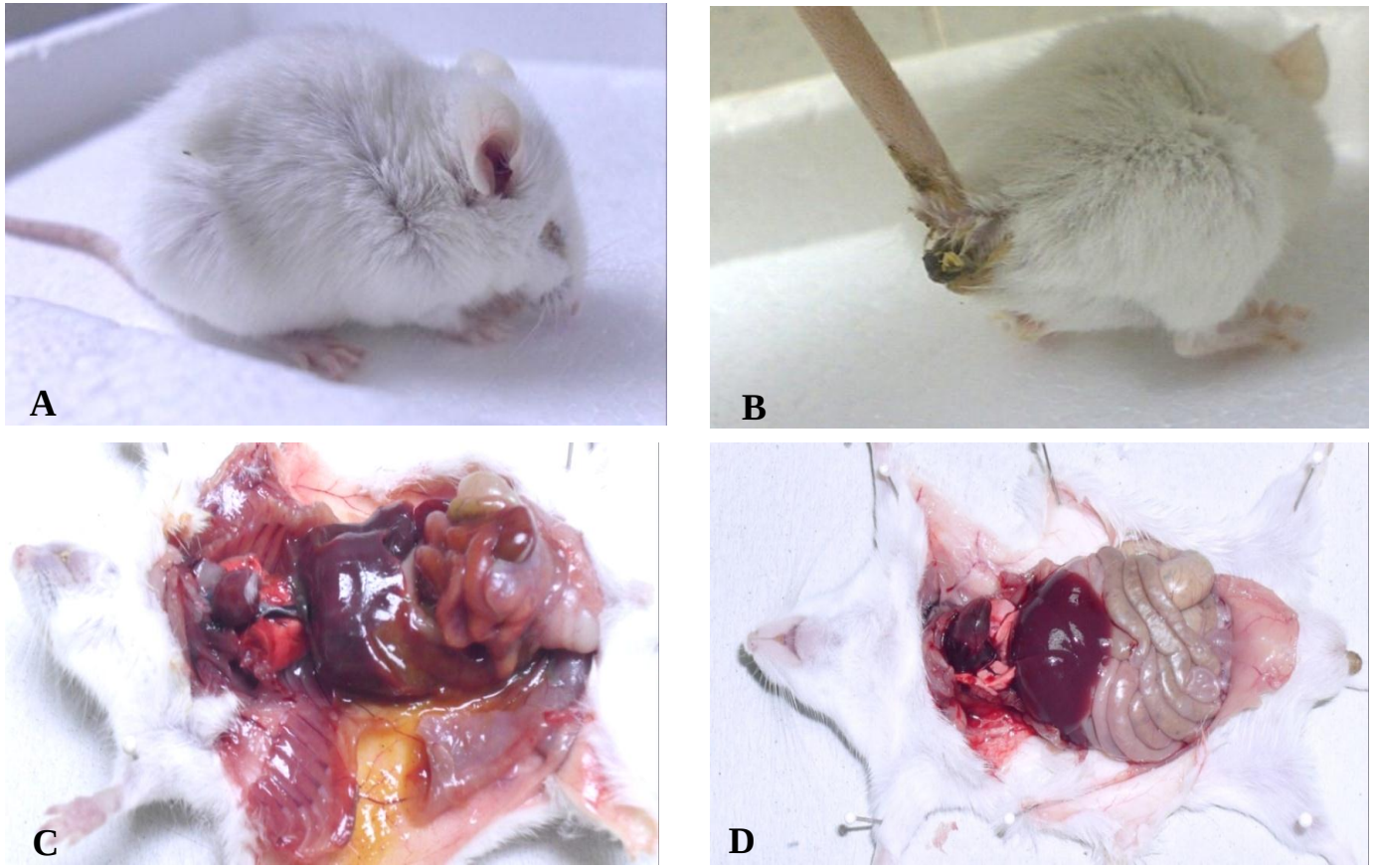


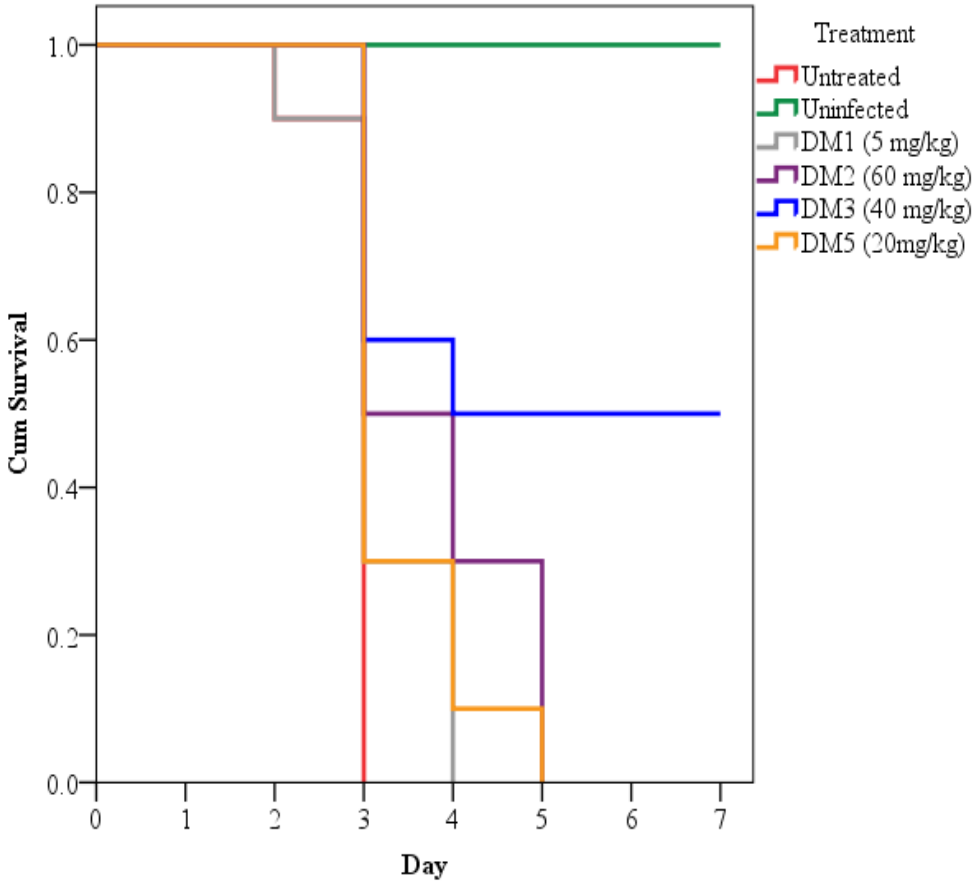
Figure 3.13: Images showing the physical abnormalities observed in pneumococcal systemic infection model of mice. Clinical symptoms of infection were observable beginning day 2 postinfection including (A) ruffled fur, hunched posture, presence of fluid around the orbital regions, and (B) difficulty in defecation. Upon necropsy, the various organs of (C) the systemically infected mouse especially lungs were presented with exudates, swelling, and redness denoting severe inflammations as compared to the (D) uninfected control.

Table 3.23: Number of mice survived at day 4 and day 7 (n = 3) following DMs treatment in two pneumococcal infection models induced by a PRSP strain.

Route	Peptide	Dose (mg/kg)	No. of mice survived			
			Systemic infection		Pneumonia	
			Day 4	Day 7	Day 4	Day 7
IP	DM1	5	1/3	0/3	0/3	0/3
	DM2	60	1/3	0/3	0/3	0/3
	DM3	40	2/3	2/3	0/3	0/3
	DM4	5	0/3	0/3	0/3	0/3
	DM5	20	1/3	0/3	0/3	0/3
	Untreated	Water	0/3	0/3	0/3	0/3
	Uninfected	Water	3/3	3/3	3/3	3/3
SC	DM1	100	0/3	0/3	0/3	0/3
	DM2	100	0/3	0/3	0/3	0/3
	DM3	100	0/3	0/3	0/3	0/3
	DM4	100	0/3	0/3	0/3	0/3
	DM5	100	0/3	0/3	0/3	0/3
	Untreated	Water	0/3	0/3	0/3	0/3
	Uninfected	Water	3/3	3/3	3/3	3/3
IN	DM1	20	0/3	0/3	0/3	0/3
	DM2	20	0/3	0/3	0/3	0/3
	DM3	20	0/3	0/3	0/3	0/3
	DM4	20	0/3	0/3	0/3	0/3
	DM5	20	0/3	0/3	0/3	0/3
	Untreated	Water	0/3	0/3	0/3	0/3
	Uninfected	Water	3/3	3/3	3/3	3/3

Highlighted in yellow: Treatments conferring enhanced survival postinfection as compared to untreated control postinfection - to be further tested for therapeutic efficacy using group of 10 mice.

Cumulative survival of lethal pneumococcal systemic infection mice treated with DMs (n = 10)

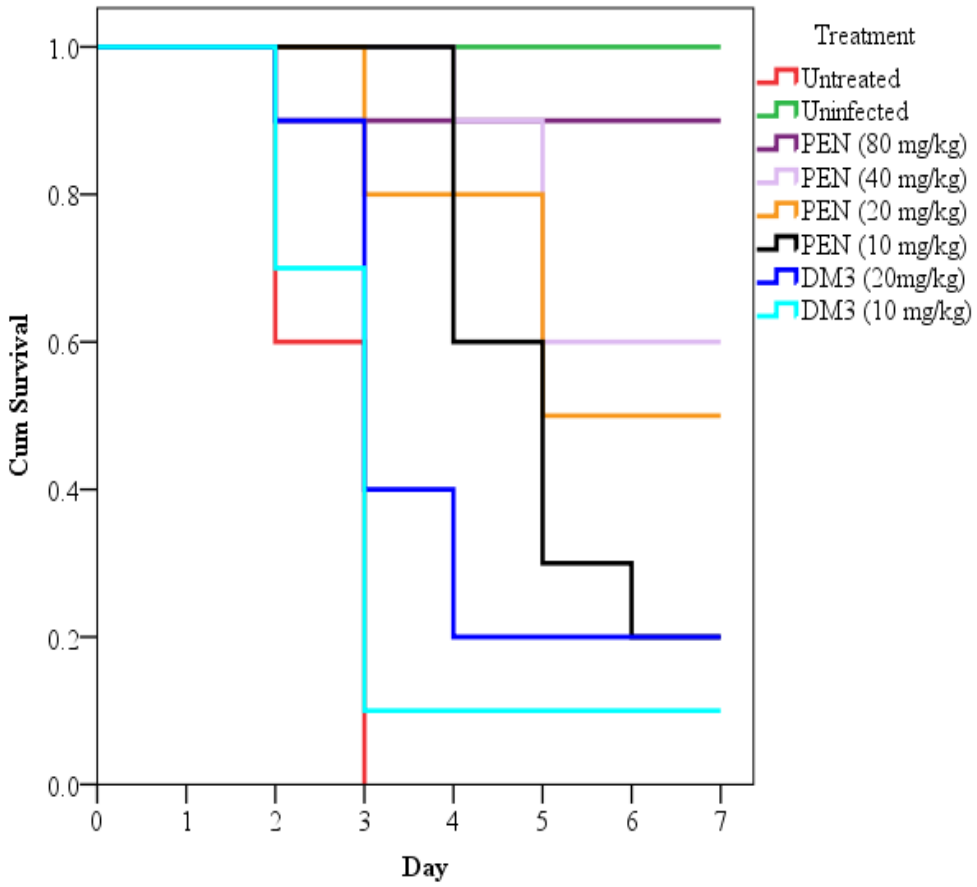


Treatment	Dose (mg/kg)	Survival %[a]	Significance[b]
DM1	5	0	0.159
DM2	60	0	0.009
DM3	40	50	0.004
DM5	20	0	0.045
Untreated		0	-
Uninfected		100	-

[a]Survival rate at day 7.
[b]Kaplan-Meier with log-rank (Mantel-Cox) test for each treatment vs untreated group

Figure 3.14: Survival function analysis of mice with lethal pneumococcal systemic infection treated with DM1, DM2, DM3, and DM5 for three doses regimen (2 hrs, 12 hrs, 24 hrs) via IP route postinfection. Statistical analysis was performed for each treated group versus untreated group using Kaplan-Meier with log-rank test (Mantel-Cox). DM3 treatment at 40 mg/kg conferred significant survival function (p = 0.004) with 50% survival rate up to day 7 postinfection. Mice treated with DM2 (p = 0.009) and DM5 (p = 0.045) showed statistically significant enhanced survival although no survival was noted up to day 7 postinfection.

Cumulative survival of lethal pneumococcal systemic infection mice treated with PEN and low doses DM3 (n = 10)



Treatment	Dose (mg/kg)	Survival %[a]	Significance[b]
PEN	80	90	< 0.001
	40	60	< 0.001
	20	50	< 0.001
	10	20	< 0.001
DM3	20	20	0.019
	10	10	0.424
Untreated		0	-
Uninfected		100	-

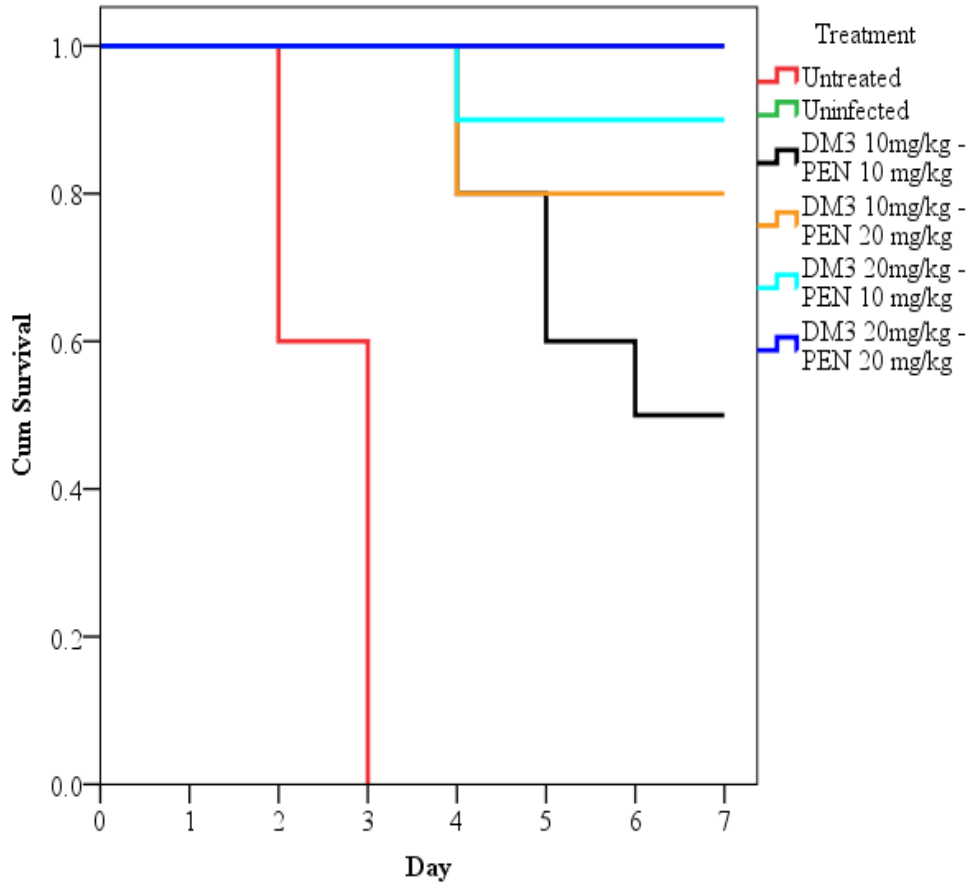
[a]Survival rate at day 7.
[b]Kaplan-Meier with log-rank (Mantel-Cox) test for each treatment vs untreated group

Figure 3.15: Survival function analysis of mice with lethal pneumococcal systemic infection treated with penicillin and low doses DM3 for three doses regimen (2 hrs, 12 hrs, 24 hrs) via IP route postinfection. Statistical analysis was performed for each treated group versus untreated group using Kaplan-Meier with log-rank test (Mantel-Cox). Penicillin treatment at 10 mg/kg, 20 mg/kg, 40 mg/kg, and 80 mg/kg showed highly significant survival function ($p < 0.001$) with 20%, 50%, 60%, 90% survival rates up to day 7 postinfection. While for lower graded DM3 doses of 10 mg/kg and 20 mg/kg, there were 10% ($p = 0.424$) and 20% ($p = 0.019$) survival rates, respectively.

3.2.5. Therapeutic synergism of DM3 in combination with penicillin

Among the hybrids, standalone treatment with DM3 at 40 mg/kg was found to confer significant survivability to mice lethally challenged by a PRSP strain systemically. To evaluate the therapeutic synergism of DM3 in combination with the standard antibiotic penicillin, lower doses of DM3 (10 mg/kg, 20 mg/kg) and penicillin (10 mg/kg, 20 mg/kg) were tested using the same systemic infection model in four treatment formulations: $DM3_{10}$ - PEN_{10} (10 mg/kg of DM3 and 10 mg/kg of penicillin), $DM3_{10}$ – PEN_{20} (10 mg/kg of DM3 and 20 mg/kg of penicillin), $DM3_{20}$ - PEN_{10} (20 mg/kg of DM3 and 10 mg/kg of penicillin), and $DM3_{20}$ – PEN_{20} (20 mg/kg of DM3 and 20 mg/kg of penicillin). While treatment with low doses of standalone DM3 and penicillin (Figure 3.15) conferred only minimal survivability whereby DM3 at 10 mg/kg and 20 mg/kg protected 10% and 20% of mice and penicillin at 10 mg/kg and 20 mg/kg protected 20% and 50% of mice, respectively. Testing using group of 10 mice showed that $DM3_{10}$ - PEN_{10}, $DM3_{10}$ – PEN_{20}, $DM3_{20}$ - PEN_{10}, and $DM3_{20}$ – PEN_{20} treatments conferred 50%, 80%, 90%, and 100% survival ($p < 0.001$) in mice lethally infected with pneumococcal systemic infection at day 7 postinfection (Figure 3.16). These survival rates were higher than the sum of the survival rates for the respective peptide/antibiotic in its standalone form by 20%, 20%, 50%, and 30%, respective for $DM3_{10}$ - PEN_{10}, $DM3_{10}$ – PEN_{20}, $DM3_{20}$ - PEN_{10}, and $DM3_{20}$ – PEN_{20} groups. Notably, all mice (100%) survived when DM3 and penicillin were both given in combination at 20 mg/kg. Hence, DM3 and penicillin displayed potent therapeutic efficacy in a synergistic fashion. In addition, the mice survived from the lethal infection following the treatments remained physically active with no sign of physical or behavioral abnormality.

Cumulative survival of lethal pneumococcal systemic infection mice treated with combinations of DM3 and penicillin (n = 10)



Treatment Dose (mg/kg)		Survival %[a]	Significance[b]
DM3	PEN		
10	10	50	< 0.001
10	20	80	< 0.001
20	10	90	< 0.001
20	20	100	< 0.001
Untreated			-
Uninfected		100	-

[a]Survival rate at day 7.
[b]Kaplan-Meier with log-rank (Mantel-Cox) test for each treatment vs untreated group

Figure 3.16: Survival function analysis of mice with lethal pneumococcal systemic infection treated with combinations of DM3 and penicillin at $DM3_{10} - PEN_{10}$, $DM3_{10} - PEN_{20}$, $DM3_{20} - PEN_{10}$, and $DM3_{20} - PEN_{20}$ for three doses regimen (2 hrs, 12 hrs, 24 hrs) via IP route postinfection. Statistical analysis was performed for each treated group versus untreated group using Kaplan-Meier with log-rank test (Mantel-Cox). The combination treatments showed significant (P < 0.001) survival function and 50%, 80%, 90%, and 100% survival rates were achieved with $DM3_{10} - PEN_{10}$, $DM3_{10} - PEN_{20}$, $DM3_{20} - PEN_{10}$, and $DM3_{20} - PEN_{20}$, respectively.

3.2.6. Histological examination of the pneumococcal infected mice

The histological examinations of the systemically infected mice with and without treatment are shown in Figure 3.17. Overall, the infected organs showed severe inflammation and tissue damages. Lungs represent the most severely affected organ with significant postinfection changes. As compared to the uninfected normal mice (Figure 3.17A), several pathological changes were noted in the lung of the infected control mice including high vascular congestion, highly thickened alveolar wall with foci consolidation due to excessive capillary congestion and edema, and alveolar exudates filled with inflammatory cells mainly neutrophils and macrophages (Figure 3.17B, black arrow). Heavy infiltrations of erythrocytes into the alveolar spaces and pulmonary tissues strongly indicate pulmonary hemorrhage (Figure 3.17B, blue arrow). This is of significant contrast to the uninfected mice displaying the highly aerated alveolar spaces with thin layer of alveolar wall in the normal lung. The highly congested lung can be seen left with little alveolar spaces (Figure 3.18, Lung - A). The exudative process is typical for bacterial lung infection which was *S. pneumoniae* in the current study as confirmed by plating the homogenates of the organs on blood agar. Severe damage was also seen with spleen especially at the white pulp area (Figure 3.18, spleen - A, black arrow).

In addition, the infected control mice displayed acute glomerulonephritis as observed with severe inflammation causing large areas damages and loss of glomerular structures (Figure 3.18, kidney - A, black arrow). The inflammatory cells and erythrocytes can be seen infiltrating the affected area (Figure 3.18, kidney - A, yellow and blue arrows) as compared to the normal kidney with intact glomerular structures (Figure 3.18, kidney - B). The formation of large lesions can be clearly observed in the kidney of infected control mice (Figure 3.18, kidney - A, yellow arrow). The epithelial cells surrounding the lumen can be seen damaged and are surrounded by inflammatory

cells. For brain, the presence of erythrocytes was detectable (Figure 3.18, brain – A, blue arrow) although the extent of inflammation was less significant as compared to the other organs.

For the respective treatments including DM3 at 10 mg/kg, 20 mg/kg, and 40 mg/kg as well as the combination treatments of $DM3_{10}$ – PEN_{10}, $DM3_{10}$ – PEN_{20}, $DM3_{20}$ – PEN_{10}, and $DM3_{20}$ – PEN_{20}, it was observed that although the lesions (if any), inflammatory events, and the extent of tissues damages can be found in these organs, however, the quantity and the magnitude of the damage were significantly lower than the infected control mice. For instance, all the lungs of the treated mice showed only low level vascular congestion and slight thickening of the alveolar wall even though these conditions were still detectable in the mice (Figure 3.18, lung – C to I). However, the inflammation and damages were minimal as compared to the infected lung which showed almost 90% congestion. In the liver, the epithelial cells and hepatocytes surrounding the vascular lumen can be seen damaged with large lesions (Figure 3.18, liver - A) as compared to the other groups of mice which had only small lesions with few inflammatory cells. Also, the treated mice had significantly lower lesions quantitatively in the liver as well. Similarly, the glomerular filtration unit and the proximal/distal convoluted tubules in the kidney of treated mice appeared normal with no major inflammation/lesions. Moreover, there was no hemorrhage or only minimal in the treated mice as compared to the infected control mice.

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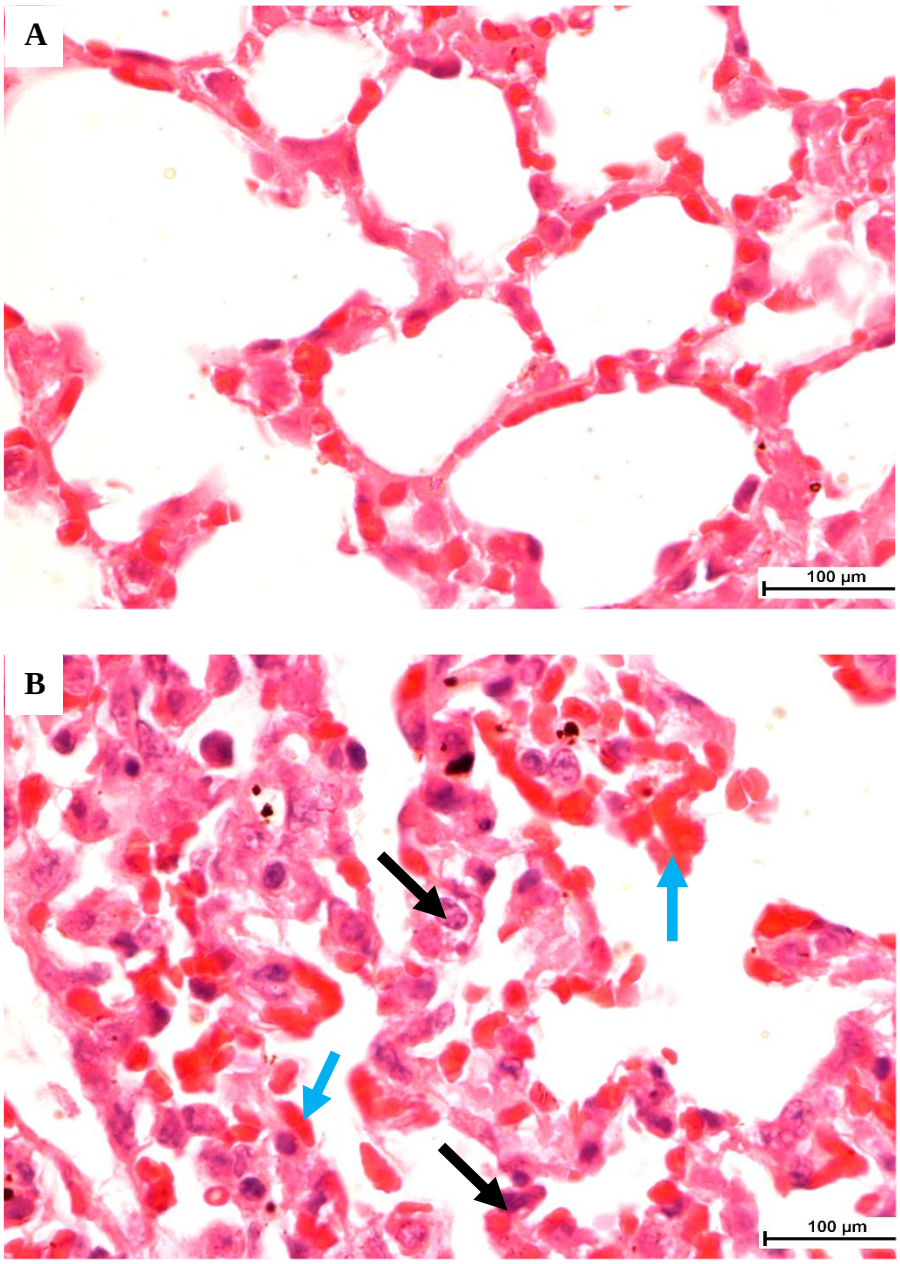

Figure 3.17: Histology of lung tissues from mice infected lethally by *S. pneumoniae*. As compared to the (A) uninfected control, the lung from (B) infected control displayed significant changes with thickening of alveolar wall and excessive alveolar exudates filled with inflammatory cells mainly the neutrophils (black arrow). The infiltrations of erythrocytes into the alveolar wall and alveolar spaces strongly indicate pulmonary hemorrhage. Magnification at 1000X, H & E staining. Bar indicates 100 µm.

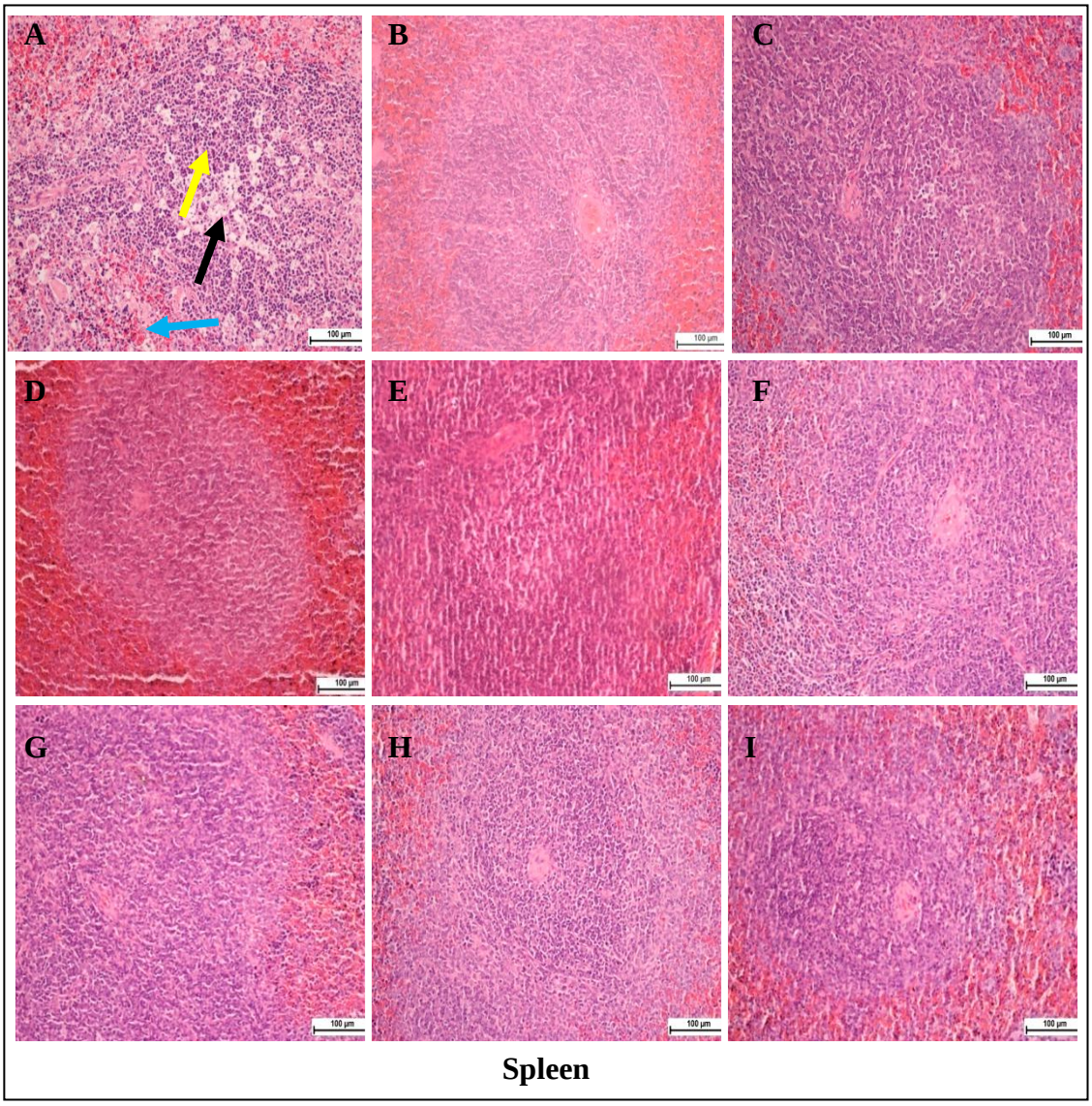

Figure 3.18: Histology of five major organs of mice lethally infected by *S. pneumoniae*.

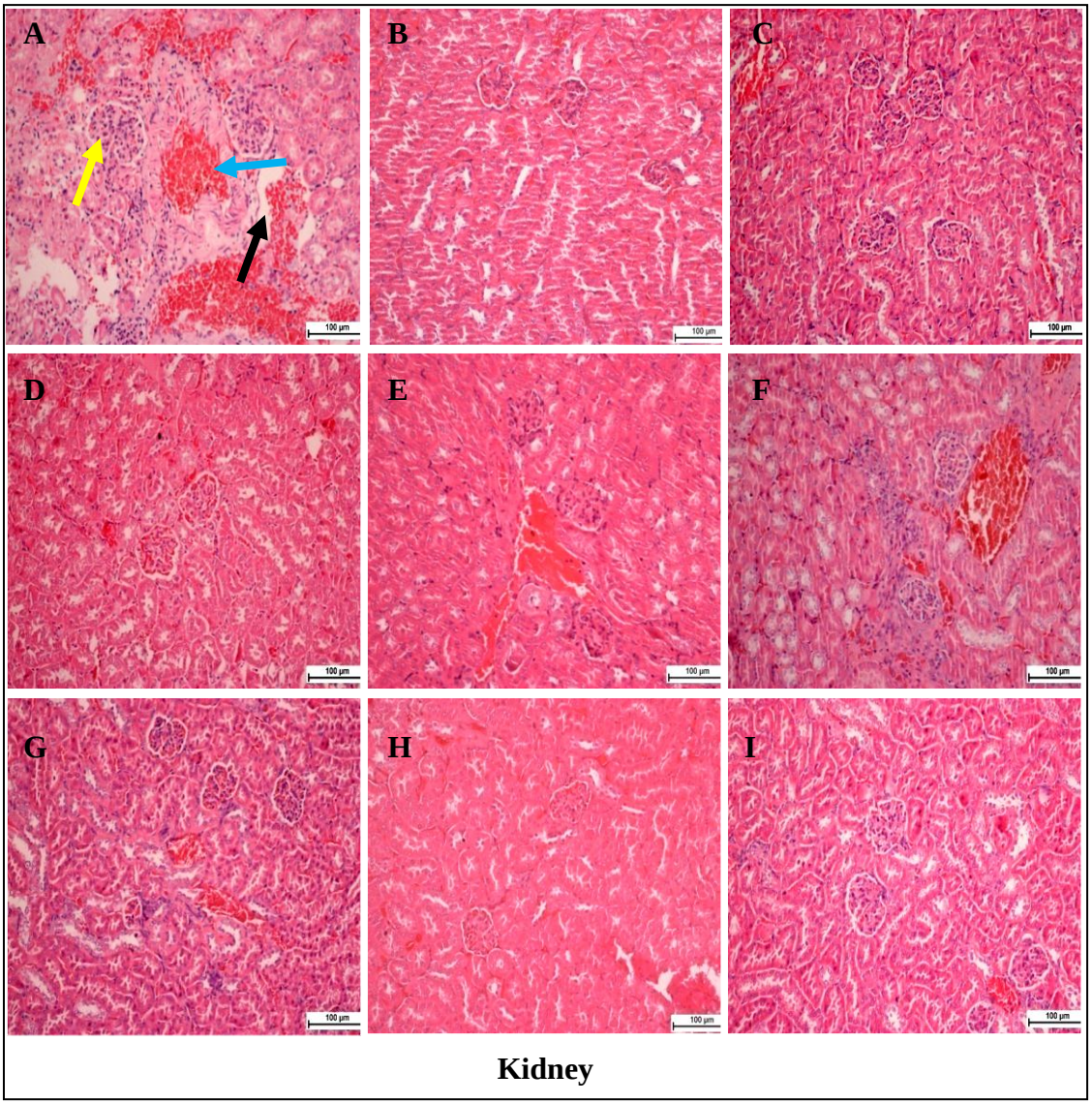

Figure 3.18 (continued): Histology of five major organs of mice lethally infected by *S. pneumoniae*.

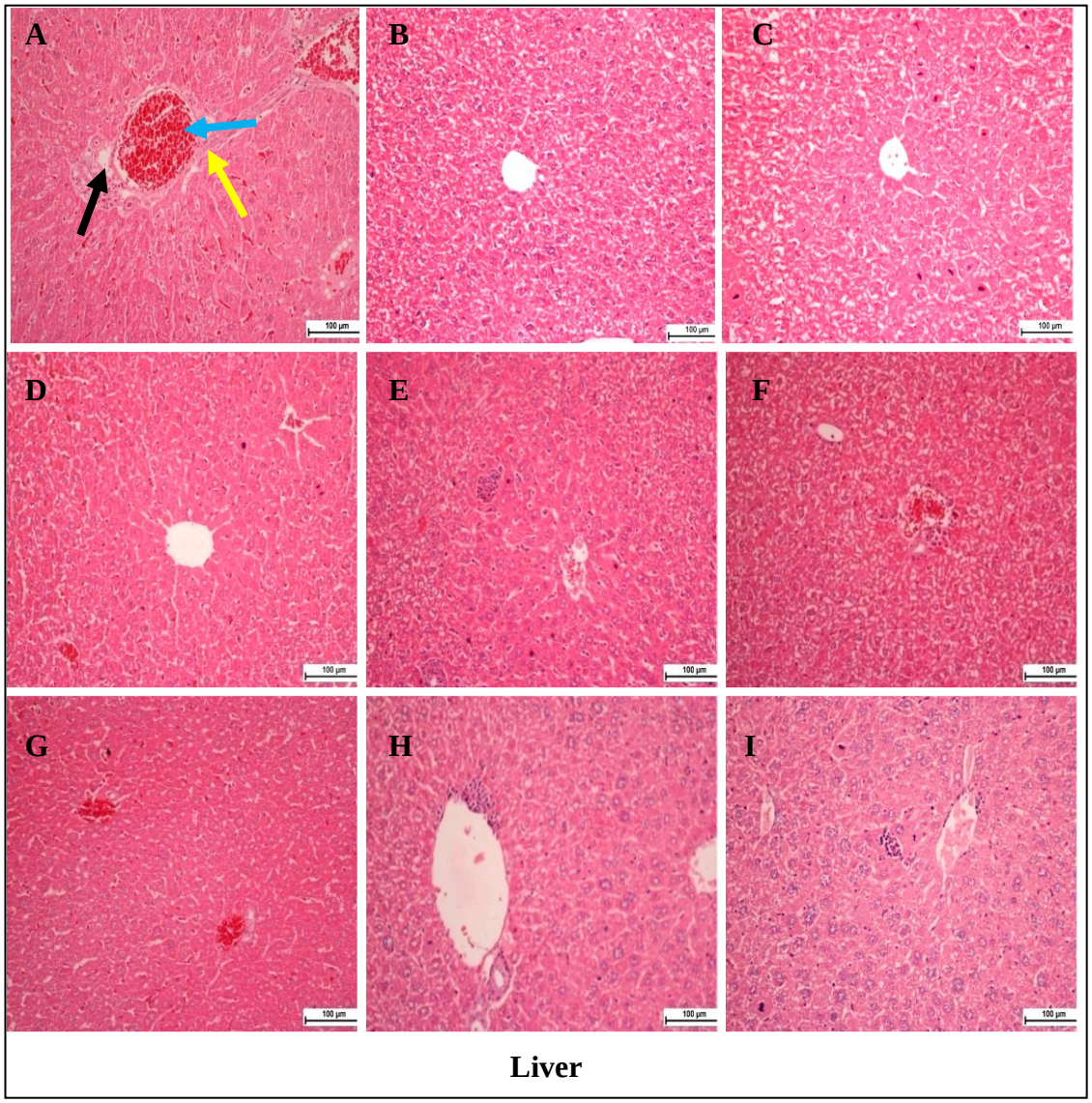

Figure 3.18 (continued): Histology of five major organs of mice lethally infected by *S. pneumoniae*.

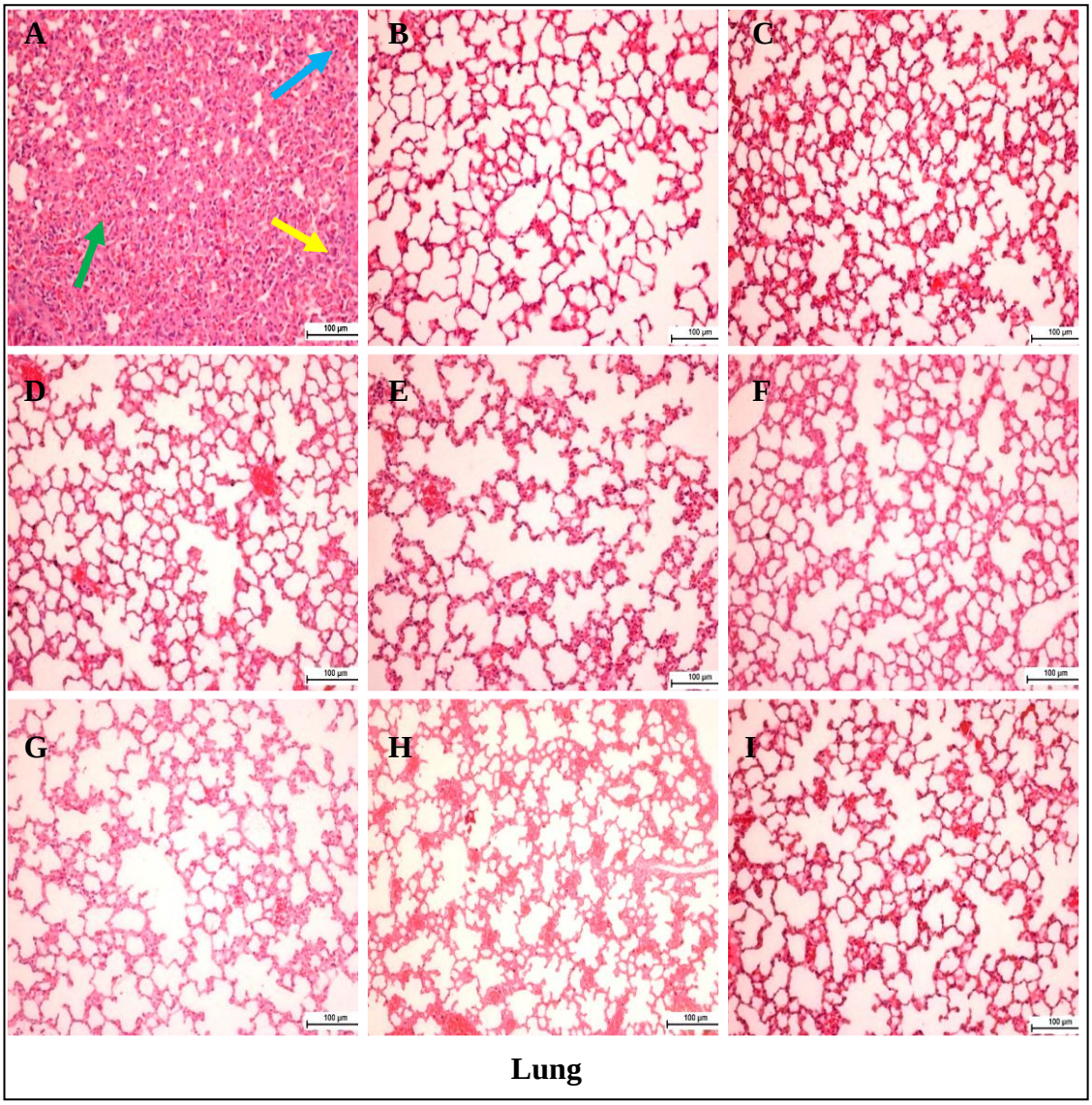

Figure 3.18 (continued): Histology of five major organs of mice lethally infected by *S. pneumoniae*.

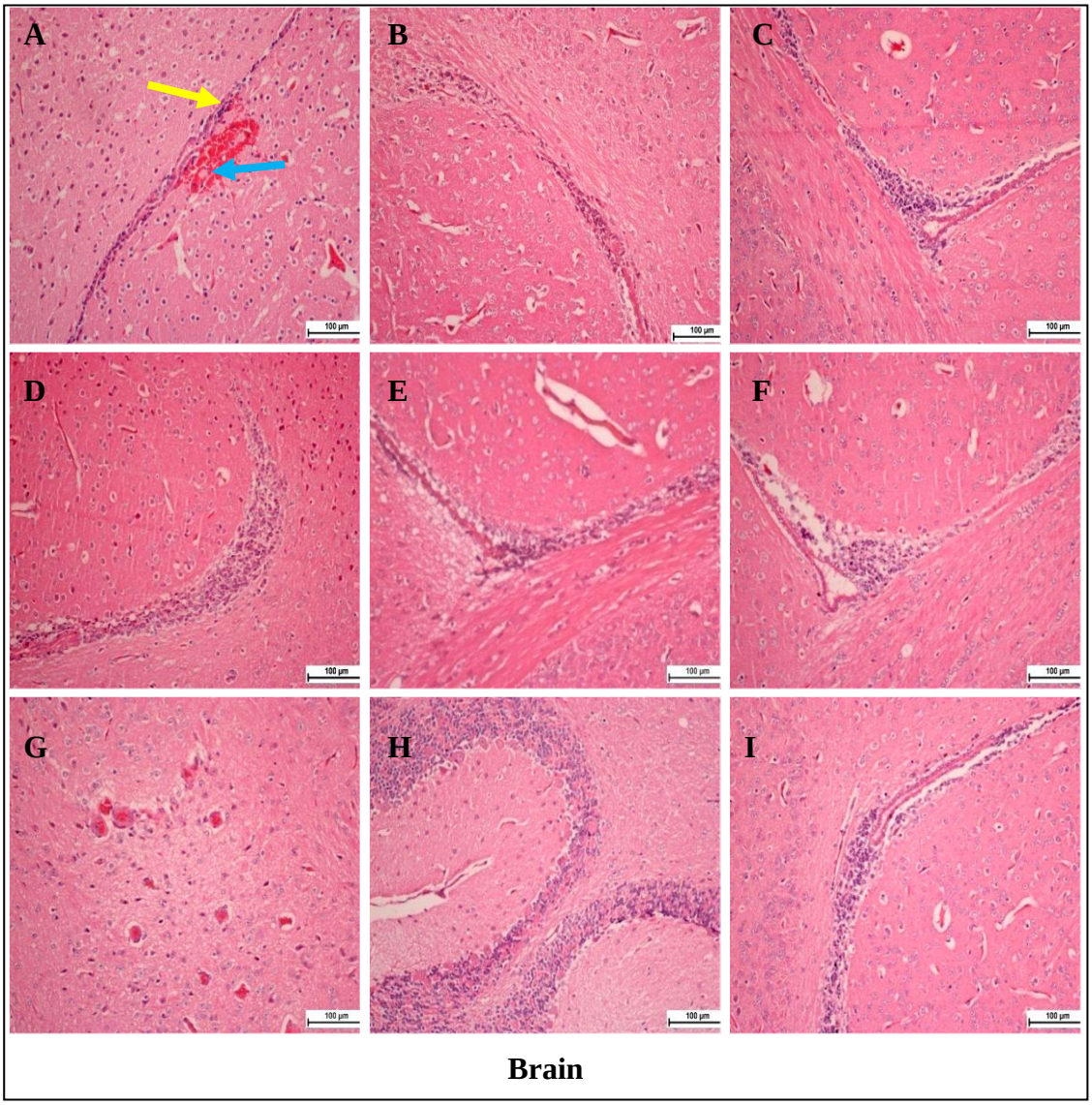

Figure 3.18 (continued): Histology of five major organs of mice lethally infected by *S. pneumoniae*. Images showing tissues from mice receiving (A) infected control, (B) uninfected control (normal), (C) DM3 at 10 mg/kg, (D) DM3 at 20 mg/kg, (E) DM3 at 40 mg/kg, (F) $DM3_{10}$ – PEN_{10}, (G) $DM3_{10}$ – PEN_{20}, (H) $DM3_{20}$ – PEN_{10}, and (I) $DM3_{20}$ – PEN_{20}. Organs from the infected control mice showed multiple lesions (yellow arrow), infiltration of erythrocytes andor vascular congestion (blue arrow), and damage of tissue structure (black arrow). Heavy alveolar congestion in the lung can be observed (green arrow). These histoplogical changes in the treated mice were significantly minimal as compared to the inected control mice. Magnification at 200X, H & E staining. Bar indicates 100 µm.

CHAPTER 4

DISCUSSION

4.1. Pneumococcal serotype distribution and penicillin susceptibility

The on-demand information on the antibiotic susceptibility and serotype distribution of *S. pneumoniae* among the local population is relatively limited. A few studies conducted previously have reported different findings, probably due to the study designs, different geographical distributions and hence the populations involved in the respective studies (Cheong *et al.*, 1988; Choo *et al.*, 1990; Hussain *et al.*, 1998; Jamal *et al.*, 1987; Lim *et al.*, 2007; Rohani *et al.*, 1999b; Tee, 1993). For these reasons, comparing study outcomes reported by other researchers is not always feasible due to the variations in study design. Moreover, the epidemiological data available on the pneumococcal carriage and infections in Malaysia throughout the years is extremely scarce (Le *et al.*, 2012).

From the current study, the incidence of penicillin nonsusceptible *S. pneumoniae* (PNSP) were remarkably high which constituted half of the total pneumococcal isolates obtained (Le *et al.*, 2011b). Our study clearly addresses the overwhelming penicillin resistance among the local isolates of *S. pneumoniae* in recent years. Such drastic increment especially the nonsusceptible strains could mean further reduction in treatment outcomes for patients infected with pneumococcal diseases. To evaluate the temporal fluctuations in pneumococcal penicillin resistance among the Malaysian population, we compiled all findings reported by various research groups over 24 years from year 1984 – 2009 (except 2001 and 2004) regardless of the study designs (Cheong *et al.*, 1988; Desa *et al.*, 2003; Le *et al.*, 2011b; Rohani *et al.*, 1999a; Rohani *et al.*, 1999b; Rohani *et al.*, 2011). To have the same penicillin minimum inhibitory concentrations (MICs) interpretative breakpoint among these studies (susceptible, $\leq$ 0.06 µg/ml, intermediate, 0.12 - 1.0µg/ml, resistant, $\geq$ 2.0µg/ml), the penicillin (oral) criteria was followed for Rohani *et al.* (2011). The major trend observed was that pneumococcal susceptibility to penicillin has decreased over the period from 1984 to 2007 followed by a slight increase in year 2008/2009 (Figure 4.1). In the mid 1980s,

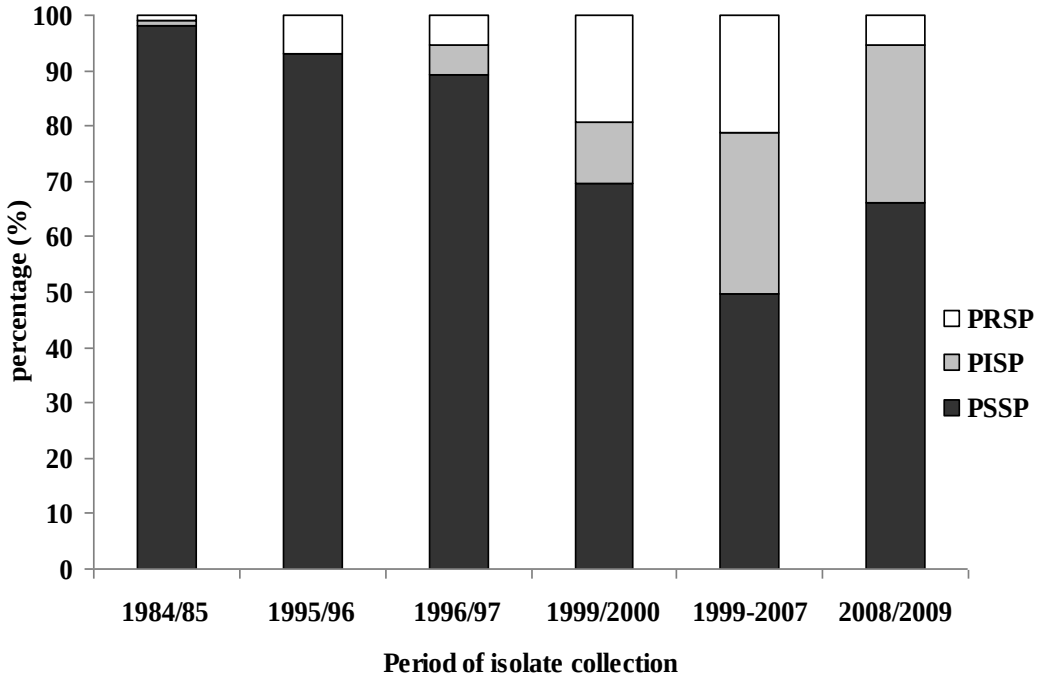

Figure 4.1: Penicillin susceptibility of Malaysian pneumococci over 24 years from 1984 – 2009 (Le *et al.*, 2012).

PNSP strains made up only less than 5% of the reported cases. However, the situation continued to change with consistent expansion of PNSP strains in the mid 1990s. Beginning year 2000, PNSP constituted 30% - 50% of the Malaysian isolates and penicillin-intermediate *S. pneumoniae* represent the dominant portion of PNSP which remained stable throughout the years. The increased proportion of PNSP isolates can be attributed to the underlying expansion of PISP as well as penicillin-resistant *S. pneumoniae* (PRSP).

A similar trend of penicillin susceptibility was also observed when comparing studies with similar study design as ours which covers patients of all age groups and isolates from various clinical sites. The percentage of PNSP had increased substantially from 10.9% (Rohani *et al.*, 1999a) to 31.0% (Desa *et al.*, 2003) within four years time and further increased to 50.3% based on current study followed by a drop to 33.9% in year 2009 (Rohani *et al.*, 2011). Additionally, the proportion of PRSP had also

increased from 5.5% (Rohani *et al.*, 1999a) to 20.0% (Desa *et al.*, 2003) and 21.2% (current study) which later decreased to 5.44% (Rohani *et al.*, 2011). Since both studies by Rohani *et al*. included pneumococcal isolates from multiple hospitals while those by Desa *et al*. and our current data were based in University Malaya Medical Centre (UMMC) only, we speculate that the rate of PRSP in Malaysia is still considerably low (< 6.0%) but in densely populated areas especially the states of Selangor and the nearby Kuala Lumpur, the PRSP isolates could be encountered at a rate of four times higher than other less-densely populated areas.

The current study also investigated on the pneumococcal serotype epidemiology and its relationships with various underlying factors such as pneumococcal penicillin susceptibility, invasiveness of isolates, and the age of patients. Children < 5 years old and the elderly adults were the major age group frequently infected with pneumococcal diseases. The respiratory tract remains as the most important clinical site for pneumococcal infections where large numbers of isolates were recovered from nasopharyngeal site (NP), sputum, and tracheal secretions. The prevailing serotype 19F dominated as high as one third of the pneumococcal isolates among the local populations followed by serotypes 23F, 1, 6A/6B were the predominant serotypes (Le *et al.*, 2011b). Serotypes 19F and 23F alone constituted approximately half of all serotypes. Although serotype 19F isolates were from rather noninvasive sites, of concern is that majority (78.6%) of the serotype 19F isolates was nonsusceptible to penicillin and half of them were penicillin-resistant. Serotype 19F was essentially the most abundant serotype particularly among younger age patients. On the other hand, serotype 19A was associated with penicillin-susceptible *S. pneumoniae* (PSSP). This serotype showed high invasive tendency to cause invasive pneumococcal disease at which 75.0% of the isolates were obtained from blood and was commonly affecting the 5 – 59 years old age group. Furthermore, it was noticed that the pool of serotypes became less diversified

with increasing penicillin resistance. This means that only certain serotypes were in the PISP and PRSP groups. Serotypes 19F, 23F, and 6A/6B were present persistently in all three susceptibility groups. On the other hand, serotype 34 and 9V/9A were present neither in PSSP nor PISP but only in PRSP group and this suggests the tendency in developing penicillin resistance might be serotype-specific.

The recent five year trend in serotype distribution among the Asian countries was evaluated in relation to the finding obtained from the current study (Le *et al.*, 2011a). The distribution of prevailing serotypes in Malaysia was mainly heptavalent pneumococcal conjugate vaccine (PCV7) vaccine serotypes which was also comparable to the neighboring country Thailand (Baggett *et al.*, 2009; Srifeungfung *et al.*, 2010) and the East Asian countries more distantly situated from Malaysia such as China (Chen *et al.*, 2010; Liu *et al.*, 2008b; Xue *et al.*, 2010; Yao *et al.*, 2011; Zhang *et al.*, 2011; Zhou *et al.*, 2011), Taiwan (Hsieh *et al.*, 2008), South Korea (Baek *et al.*, 2011; Choi *et al.*, 2008; Song *et al.*, 2009), and Sri Lanka (2008). West Asian countries/territories such as Oman (Al-Yaqoubi & Elhag, 2011) and Israel (Somech *et al.*, 2011) also found to have seroepidemiology resembling those for the Malaysian population. This pattern of serotype distribution was also relatively different from Singapore (Hsu *et al.*, 2009; Jefferies *et al.*, 2011), Hong Kong (2011; Ho *et al.*, 2011; Hon *et al.*, 2010; Ip *et al.*, 2007), Japan (Chiba *et al.*, 2010; Hotomi *et al.*, 2008; Oishi *et al.*, 2011; Sakai *et al.*, 2011; Suzuki *et al.*, 2010). The incorporation of PCV7 vaccine into the childhood immunization program has led to the reports of reduced vaccine serotypes in the United States (Farrell *et al.*, 2007; Grijalva *et al.*, 2007; Kaplan *et al.*, 2004; 2010), Germany (Ruckinger *et al.*, 2009), Norway (Vestrheim *et al.*, 2008), France (Dubos *et al.*, 2007; Lepoutre *et al.*, 2008), Canada (Kellner *et al.*, 2009), and Australia (Roche *et al.*, 2008). The nonvaccine serotypes are emerging considerably through serotype replacement in place of the reduction in vaccine serotypes. For instance, the predominant serotypes

among children with invasive pneumococcal disease (IPD) were serotypes 19A, 7F, 3, and serogroup 15 (McNeil *et al.*, 2009). Despite PCV7 vaccine serotypes being the predominant serotypes prior to the introduction of PCV7 vaccine in year 2002 in Canada, serotypes 5, 8, 3, 14, and 22F have now been the most common serotypes based on distribution from 2002 – 2007. Pneumococcal conjugate vaccines have not been commonly in use in Asian countries despite Hong Kong, Singapore, and Japan having included PCV7 vaccine in their routine childhood immunization. This late implementation (after year 2009) means that it is still too early to assess the significant changes in serotype distribution and continued surveillances are needed.

To evaluate the potential efficacies of PCV7 vaccination among the Malaysian population, serotypes covered under the vaccine were analyzed. This PCV7 vaccine covers serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F and also provides protection against those serotypes of the same serogroup as the formulated types. As the prevailing serotypes have been formulated under PCV7 vaccine, it was determined that 87.5% PRSP (28/32), 79.5% PISP (35/44), and 45.3% PSSP (34/75) are covered by the current PCV7 vaccine. PCV7 is thus predicted to have 64.2% of coverage (97/151). Therefore, effectiveness of PCV7 vaccine in our community would be encouraging. Towards reducing the morbidity and mortality associated with pneumococcal infections, it is highly recommended that PCV7 vaccine to be incorporated in the regular vaccination scheme for children in Malaysia. This will help to reduce the incidence directly in those young children as well as indirectly as a result of herd community effect. Children with certain chronic diseases or preexisting medical conditions which put them at high risk of developing IPD should be considered for the vaccination. This includes sickle cell disease, asplenia, immunosuppression, renal disease, cerebrospinal fluid leaks, HIV infection, and others. Nonetheless, it is difficult to predict the shift in serotype prevalence in the future. Thus, continuing surveillance of pneumococcal carriage and

diseases-related cases will help to characterize the effectiveness of PCV7 vaccine and monitoring of disease burden due to nonvaccine serotypes are certainly needed. This information will also provide the local regulatory body to recommend suitable vaccines to be used.

It is particularly interesting to note how these serotypes are related to penicillin-resistance and the associated intracellular alterations that lead to the changes against penicillin stress. Many other minor and non-prevalent serotypes have also been detected among all three groups. This may be due to variations in the geographical area, disease, and age groups of the patients. However, the high prevalence of penicillin resistance in this study suggests the rapid spread of resistant clones among pneumococci strains in this region.

Since pneumococci has the ability to switch serotypes by horizontal transfer recombination and other genetic events, it is important to monitor the frequency of the serotype exchanges in order to predict long term efficacy of new vaccines. Therefore continuous monitoring of antimicrobial resistance and serotype distribution of *S. pneumoniae* is important for disease management and various disease-control policies among the Malaysian population. The current study supports the need for a long term surveillance program and appropriate vaccination strategy towards prevention and control of pneumococcal infections. We hope that current study will contribute to the regional body of knowledge on *S. pneumoniae* serotypes distribution in Malaysia, such as the Asian Strategic Alliance for Pneumococcal Diseases Prevention (ASAP) in align to their mission on control and contain pneumococcal diseases in Asian countries.

4.2. Designing antimicrobial peptides with improved antimicrobial activity

The increase in reports of pneumococcal infections associated with antibiotic resistant strains has prompted for better disease-control and prevention strategies. Newer

generation antibiotics with high antimicrobial potential especially against the antibiotic resistant *S. pneumoniae* have been the major interest. Synthetic derivatives of antimicrobial peptides (AMPs) with enhanced antimicrobial activity have been increasingly documented. The antimicrobial potential of AMPs is frequently recognized by a set of common rules, with multiple cationic charges and high hydrophobicity representing the principal factors for AMPs to be actively antimicrobials (Chen *et al.*, 2007; Dathe *et al.*, 1997; Leptihn *et al.*, 2010; Yin *et al.*, 2012; Yu *et al.*, 2010). The cationicity of AMPs represents the primary factor governing the initial contact with the oppositely charged bacterial surface via electrostatic interactions to facilitate subsequent actions (Dathe *et al.*, 2001; Matsuzaki *et al.*, 1997a; Yeaman & Yount, 2003). Furthermore, the presence of specific amino acids allocated at the precise position in the peptide chain is also crucial for the expression of antimicrobial activity. This probably happens as the amino acids, each with unique functional group, possesses a variety of physicochemical properties of great variety and the allocation of these amino acids at precise positions in the peptide chain thus ensuring the structural integrity and stability of AMPs required for specific interaction with the targets. Moreover, this also implies that there exist limitations in the maximal antimicrobial activity of any given peptide with definite chain length due to the limitation in the number of possible sequence combinations. For these reasons, systematic alterations of specific amino acids and the physicochemical properties of AMPs represent a straightforward yet effective approach towards improving the antimicrobial activity of AMPs. Nevertheless, such considerations should be AMPs family-specific as it is unrealistic to expect two entirely unrelated AMPs families with high sequence diversity but which possess similar physicochemical properties to exhibit identical antimicrobial activity based solely on the physicochemical values without considering its unique sequence identity that defines the specific antimicrobial activity of the AMPs.

The primary focus of this study was to design novel designed AMPs with high potency against *S. pneumoniae*, in particular, the penicillin-resistant isolates. The carefully designed peptides displayed widely different level of antipneumococcal activity. Even so, several common features were noted whereby the pneumococcal-active designed AMPs including DAMP7, DP71, DP72, and especially the highly potent DM1 – DM5 were able to exert antipneumococcal activity irrespective of the penicillin susceptibility of the isolates. These designed AMPs also exhibited strong pneumocidal effects with significantly higher rates than the conventional antibiotic penicillin. Interestingly, the co-presence of these designed AMPs were found to enhance the antipneumococcal activity of penicillin in one way or another in a synergistic manner even against the penicillin-resistant *S. pneumoniae*. Hence, the potential application of these designed AMPs as the combination therapeutic agents to support the course of penicillin treatment are encouraging. Moreover, the antipneumococcal synergism produced with the different peptide-peptide pairs involving DM5 with DM1, DM2, DM4 and DAMP7 and DM3 with DAMP7 pairs indicate that they were able to synergize the inhibitory effects of the specific paired designed AMPs.

Besides, the potential applications of the DMs can be extended to other common bacterial infections owing to their broad spectrum antibacterial activity. This spectrum of activity did vary with the respective DMs. The comparable effective ranges against pneumococci and against other bacteria suggest that the dose range to be used against pneumococci will be as effective against multiple bacteria as well. Interestingly, these hybrids were essentially non-hemolytic as with DM1, DM4, and DM5 or at the minimal level only as with DM2. Although DM3 was the most hemolytic peptide among other DMs, the level was considerably lower than the template DAMP7 by as much as 40% at 250 μg/ml. Besides, the strong inhibitory potency of DP73 specifically against *S. aureus* suggested that DP73 could be a potential novel antistaphylococcal agent.

The step-wise procedures in designing the first group peptides based on the natural AMPs and the subsequent redesigning of the second group peptides are illustrated in Figure 4.2. The procedures in generating the five DMs by fragment hybridizations of DAMP6 and DAMP7 has been shown in Figure 3.3. Upon analyzing into the relationship between physicochemical properties and *in vitro* antipneumococcal activity of 1HR1-derived designed AMPs, net charge (NetC) of +5 and above appeared to be the prerequisite threshold to initiate antipneumococcal activity in this peptide series. This is based on the notions that both the 1HR1 (+4) and DP73 (+3) which displayed no antipneumococcal activity have lower NetC as compared to the pneumococcal-active peptide DAMP7 (+5). This also explains the high positive charges of both DP71 (+7) and DP72 (+7) probably contribute to the increase in antipneumococcal activity of peptides over DAMP7. However, whether increasing the NetC beyond +7 would further produce additional antipneumococcal activity is yet to be determined. The importance of the cationicity window for the optimal expression of antimicrobial activity of AMPs had also been documented (Dathe *et al.*, 2001; Jiang *et al.*, 2008; Tossi *et al.*, 2000). V13K analogs with NetC of < +4 were found to be inactive but analogs with higher NetC from +4 to +10 were correlated well with increasing antimicrobial activity although drastic increment in hemolytic activity was also noted at the level of > +8. Therefore, Giangaspero *et al*. had suggested the optimal cationicity window to be +4 - +8 for this particular peptide family. In addition, the cationicity window of between +4 to +6 are commonly observed among the natural AMPs (Giangaspero *et al.*, 2001). In another study, the synthetic analogs of magainins 2 with NetC up to +5 displayed increasing antimicrobial activity (Dathe *et al.*, 2001). However, further increasing the NetC above +5 to +7 had no advantageous antimicrobial effects but the hemolytic activity levels became prominent.

Natural AMP	Sequence
1VM4	GLFDIVKKLVSDF
1HR1	ILAWKWAWWAWRR

First round

Natural AMP	Sequence	Designed AMP	Sequence
1VM4	GLFDIVKKLVSDF	DAMP6	GLFDIWKKLVSDF
1HR1	ILAWKWAWWAWRR	DAMP7	ILWWKWAWWRWRR

Second round (redesigned)

Peptide	Sequence generated		
	1st cycle	2nd cycle	
DAMP6 GLFDIWKKLVSDF	GLFDIWKKKKSDF	GLFDTNKKKKSDF	DP61
	GLFDIWKKKKSDF	GTTDIWKKKKSDF	DP62
	GLFDKKKKLVSDF	GSKDKKKKLVSDF	DP63
DAMP7 ILWWKWAWWRWRR	PHWWKWAWWRWRR	PHWWKWAWWHHRR	DP71
	KHWWKWAWWRWRR	KHWWKHDWWRWRR	DP72
	ILWWLLAWWRWRR	ILWWLLAWWRWPH	DP73
1T51 ILGKIWEGIKSLF	ILGKIWEGIVLLF	ILGGDWEGIVLLF	DP51
	ILGGGWEGIKSLF	ILGGGWEGIVGLF	DP52
	ILGKILVGIKSLF	ILGKILVHHKSLF	DP53

Figure 4.2: The step-wise procedures in designing the peptides beginning from the natural AMPs, first round design which generated DAMP6 and DAMP7, and the second round redesigned which generated the DP6, DP7, and DP5 series of peptides (after two cycles of predictions).

The hydrophobicity property also affects the antimicrobial activity of peptides in a similar fashion as cationicity. As reported by Chen *et al*., an optimal hydrophobicity window (analogous to the cationicity window) which influenced the biological activity of peptides was noted (Chen *et al.*, 2007). High hydrophobicity (as well as amphipathicity) also enhanced the hemolytic activity of peptides. Based on the charge density (ChD) analysis into DAMP7, DP71, and DP72, ChD of ≥0.38 up to 12.7 would enhance the antipneumococcal potency of this peptide family.

Further supporting the findings were analysis based on the 1T51 natural AMP which had previously been reported with highly potent antibacterial activity (Lee *et al.*, 2004b). Indeed, the current study also found 1T51 to be the most potent natural AMP against *S. pneumoniae*. Nevertheless, drastic change was seen with DP5 series peptides. As observed with DP51 and DP52, decreasing the cationicity to neutral or negatively-charged also resulting in loss of potency in spite of the increase in hydrophobicity. Although DP53 has high cationicity as compared to 1T51 it still failed to produce antipneumococcal activity. Therefore, the antipneumococcal activity of 1T51 was suggested to be related to the paired relationship of both cationicity and hydrophobicity. Combining these, the possible approach towards enhancing the antipneumococcal potency of 1T51 series peptides was to elevate the positive charges while lowering the hydrophobicity of 1T51. In fact, detailed analysis into the five 13 amino acids 1T51 synthetic analogues and the antibacterial activity reported in a previous study by Lee *et al*. also supports this proposition (Lee *et al.*, 2004b).

Combining these findings, higher cationicity and lower hydrophobicity could be the favorable approach towards designing high potency designed AMPs effective against *S. pneumoniae* particularly for the indolicidin and IsCT series of peptides. Indeed, a recent study had demonstrated that multiple factors including the positive charges, charges distribution, core segment hydrophobicity, peptide helicity and others

should be in optimal balance to obtain effective AMPs with low hemolytic activity (Yin *et al.*, 2012). These findings also emphasized that the antimicrobial activity of AMPs is essentially family-specific as AMPs of different families could perform distinctively different even possessing identical physicochemical properties. Thus, designing synthetic AMPs should be based on careful consideration of the unique features associated with the AMPs family.

Additionally, previous findings by Subbalakshmi *et al.* stated that Trp was responsible for cell cytotoxicity of indolicidin peptides but served a negligible role in antimicrobial activity (Subbalakshmi *et al.*, 2000; Subbalakshmi *et al.*, 1996). This could be the possible explanation causing the elevated hemolytic activity in DAMP7, which has 8% higher Trp ratio than 1HR1. In the redesigning, lowering the DP7s Trp ratio to 38% as in 1HR1 suppressed the hemolytic activity. Further strengthening the notion was that DMs that possess 16 – 31% lower Trp ratio demonstrated significantly better hemolytic profiles than DAMP7. Interestingly, DP7 peptides were non-hemolytic as compared to 1HR1. Hence, it was suggested that other associated factors might be affecting the hemolytic activity of the peptides as well.

The natural AMPs 1HR1 and 1T51 were found to be able to significantly select the human lung epithelial adenocarcinoma A549 cell line over the human lung epithelial normal NL20 cell line. These natural AMPs might possess valuable anticancer properties that warrant further investigations. Further analysis into the cytotoxicity levels of the peptides noted that the peptides could produce drastically different cytotoxicity at high concentration which reflects the unique interactions of peptides when presented at high concentrations on human cells. In addition, the four designed AMPs including DAMP7, DM2, and DM3 appeared to be more selective against A549 than NL20 cell line.

The most surprising outcomes were seen with the five 13 amino acids hybrid peptides designed by gradual hybridization of fragments of different length of both DAMP6 and DAMP7. All five DMs displayed superior antipneumococcal activity independent of the penicillin resistance level of the isolates These pneumococci susceptible to the hybrid peptides included a variety of serotypes such as the highly prevalent serotypes 19F, 23F, and 6A/B, the less prevailing serotypes including 11A/D, 15A/F, 16F, 22F/A, 3F, and the nontypeable pneumococci. Therefore, the antipneumococcal activity of DMs was irrespective of the serotypes of *S. pneumoniae*. The rapid pneumocidal kinetic was greater than the conventional antibiotic penicillin. Besides *S. pneumoniae*, the DMs displayed broad spectrum antibacterial activity against multiple clinically important bacteria irrespective of the gram types of the bacteria.

Morphologically, the overwhelming changes observed in the DMs-treated pneumococcal cells clearly demonstrated the detrimental effects induced by DMs at the supra-MIC concentrations. Although the detailed underlying mechanisms might differ between the respective DMs, for instance, DM1 caused direct leakage of intracellular contents while DM5 caused the bulging and eventual formation of cytoplasm-containing inclusion bodies through exocytosis. Two major mechanisms that were clearly evident were cell wall and cytoplasmic membrane disruptions. Interestingly, DM3 was able to induce the collapse of cytoplasm into small inclusion bodies while the cell wall remained intact. Based on this, DM3 was suggested to have gained entry by translocation without significant impact on the cell wall to act upon the membrane. Since the pneumococcal cells were treated at supra-MIC level by the DMs thus the predominant mechanism of actions at lower concentration such as the MIC or sub-MIC level remained unclear. This is because AMPs could be membrane-active when presented at high concentration while become intracellular-targeting at low concentration or both (Friedrich *et al.*, 2000; Friedrich *et al.*, 2001).

The presence of specific amino acid residue is sometime crucial for the antimicrobial activity of the peptides. Previous study conducted by Tsai *et al*. had also determined that Lys^{13} and Arg^{22} residues were critical for the anticandidal activity of Histatin-5 whereby replacing the Lys^{13} and Arg^{22} residues with Glu^{13} and Gly^{22} residues caused a dramatic reduction in the anticandidal activity of the Histatin-5 variant m68 (Tsai *et al.*, 1996). Furthermore, Subbalakshmi *et al*. suggested that the tryptophan residues were responsible for the hemolytic activity of indolicidin analogs but has no contribution to the antimicrobial activity (Subbalakshmi *et al.*, 1996).

Based on the sequences of the hybrid peptides, it was noticed that the central WKW residues at the 6^{th} to 8^{th} position could be crucial to the antipneumococcal activity of the DMs. Substituting Lys^7 to Ala^7 of the highly potent DM3 slightly reduced the antipneumococcal activity of DM1 and DM2. Similar outcomes were noticed for DM4 and DM5 with Trp^8 to Lys^8 substitution. No significant relationship can be drawn between the antipneumococcal activity of designed AMPs and the physicochemical properties of peptides. Thus, cationicity and hydrophobicity do not seem to be the primary influencing factors for these peptides in this peptide family but the presence of certain amino acids appears to govern the antimicrobial activity of the peptides. Since both the N- and C-terminal fragments were unaltered purposefully during the redesigning of DMs, thus the sequence GLFD-X-WKW-X-RWRR-NH2 (X denotes random residue) was suggested to be the potential template to further designing better antimicrobial peptides.

Towards evaluating the actual therapeutic efficacy of peptides in biological systems of living organisms, the peptides were tested in two lethal mouse models of pneumococcal infections challenged by a PRSP strain. Based on the *in vitro* MIC findings, all five hybrid peptides were selected as they displayed the most promising antipneumococcal effects in terms of overall effective range and overall effective

percentage and the low cell toxicity levels. Among the three routes of administrations (IP, SC, IN) assessed in the lethal pneumococcal systemic infection and pneumonia models, DM3 showed great therapeutic potential with statistically significant survival function ($p \leq 0.05$) and cured 50% of the lethally infected mice as compared to the untreated vehicle without treatment intervention. Although DM2, and DM5 also showed significant survival function with delayed deaths, however, the peptides were unable to prevent lethality in the pneumococcal systemic infection model.

Apart from this, the use of two or more antimicrobial agents in combination therapy have been the alternative strategy to improve treatment outcome in the clinical setting (Caballero & Rello, 2011). This is especially valuable in patients with critical pneumococcal infections. For instance, combination therapy with both penicillin and macrolide would be considered especially when the patient is in critical pneumococcal bacteremic condition (Baddour *et al.*, 2004). The β-lactam antibiotic penicillin is commonly prescribed as empirical therapy against pneumococcal infections (Appelbaum, 1996; Klugman, 1990). However, the penicillin treatment outcome in patients has been increasingly complicated by the escalating incidences of penicillin-resistance (Jacobs, 2003; Jones, 1999). Hence, DM3 which conferred significant therapeutic efficacy and survivability to the lethally infected mice in its standalone form was further tested for *in vivo* therapeutic synergism in combination with penicillin.

Based on the *in vitro* findings, DM3 had been determined to produce antipneumococcal synergism with penicillin irrespective of the penicillin susceptibility of the isolates. Interestingly, combination of DM3 and penicillin also produced therapeutic synergism *in vivo* in the mouse model of lethal pneumococcal systemic infection. The survival rates in mice receiving the combination treatment of DM3 and penicillin at varying dosage combinations were dramatically increased by 20% - 50% as compared to the sum of the survival rates of the standalone treatment. Also, while all

untreated mice died from severe systemic pneumococcal challenge within two days postinfection, complete survival was achieved with $DM3_{20}$ – PEN_{20} formulation at day 7 postinfection and all mice showed no signs or symptoms of sickness as well as physical or behavioral abnormality. The mice were cleared from the pneumococcal challenged as evident from the clear agar plating in blood and the five major organs examined. As the synergistic survival rate of $DM3_{20}$ – PEN_{20} (30%) was expected to be higher than $DM3_{20}$ – PEN_{10} (50%) due to the higher penicillin dose (20 mg/kg), the actual synergistic survival rate has probably been underestimated as masked by the maximal (100%) survival rate achieved. These combination treatments as well as the standalone DM3 were determined to be nontoxic *in vivo* to the mice based on the whole blood and serum biochemistry parameters analysis as well as histological examination. Of note, no nephronotoxicity and hepatotoxicity detected.

The severe organ tissues inflammations and damages caused by *S. pneumoniae* in the lethally infected mice have been clearly demonstrated based on the histological findings (Figure 3.17 & Figure 3.18). This indicates that the spread and establishment of pneumococci began rapidly following the inoculation and continued for two to four days until the mice were moribund/dead. Interestingly, such histological alterations in the five organs of the mice survived from the lethal pneumococcal systemic infection following treatments with DM3 (10 mg/kg, 20 mg/kg, 40 mg/kg) and the four combination therapies with penicillin ($DM3_{10}$ - PEN_{10}, $DM3_{10}$ – PEN_{20}, $DM3_{20}$ - PEN_{10}, and $DM3_{20}$ – PEN_{20}) were minimal and was dramatically lower than the infected control mice. Although minor histological changes were still observable in these mice, this shows that the pathological sequelae caused by the highly virulent *S. pneumoniae* infection were almost unavoidable once the bacteria spread to the organs. However, the current treatments were able to significantly clear the invaded pneumococci from the

tissues and reduce these damages to the minimal level. In addition, the mice survived remained physically active with no apparent sign of physical or behavioral abnormality.

It was found that the dose of DM3 affects the therapeutic outcome much greater than the dose of penicillin. Increasing the dose of standalone penicillin by 10 mg/kg from 10 mg/kg (20%) to 20 mg/kg (50%) would enhance the protection by 30%, giving the expected survival rate of 80% in $DM3_{10}$ – PEN_{20} group over the expected survival rate of 50% in $DM3_{10}$ - PEN_{10} group and this is observed in the *in vivo* therapeutic synergism experiments. Similarly, increasing the dose of standalone DM3 by 10 mg/kg from 10 mg/kg (10%) to 20 mg/kg (20%) would enhance the protection by 10%, giving the expected survival rate of 60% in $DM3_{20}$ - PEN_{10} over $DM3_{10}$ - PEN_{10} (50%). Interestingly, the $DM3_{20}$ - PEN_{10} group recorded 90% survival rate which is 30% higher than the expected value. In other word, changes in the dose of DM3 will have greater effect than penicillin on the resulting therapeutic efficacy of the DM3-penicillin formulation. However, the importance of penicillin should not be neglected as both components are equally critical in the formulation.

Although the mechanism of antimicrobial actions of the DMs was not the focus of the current study, findings gathered based on various *in vitro* antimicrobial studies performed has led to the hypothesis that the DMs could possibly be cell wall and/or plasma membrane targeting. This is because the potent antimicrobial activities of the DMs were (1) independent of penicillin susceptibility and thus the penicillin-binding proteins which are responsible for cell wall biosynthesis processes as well as the molecular resistance mechanisms involve in penicillin resistance, (2) independent of pneumococcal serotype and thus ruled out the specific capsular polysaccharide-targeting potential of the DMs, (3) high killing rate immediately following treatment and thus should not have dependent upon the metabolic mechanisms of *S. pneumoniae* for its activity, (4) broad spectrum against variety of bacteria of both gram types in general and

thus the differences in cell wall composition have no significant interference on the DMs, and (5) images from Transmission Electron Microscopy provide clear evidence showing severe damages especially on cell wall and plasma membrane of pneumococci. Therefore, cell wall/membrane perturbation appears to be the most potential mechanism. However, we do not exclude the possibility of the involvement of other cooperative mechanisms which contribute to the antimicrobial activity of the DMs. We also speculate that in its standalone form, the biological activity of DM3 might have been interfered by various neutralizations or enzymatic reactions especially in the blood components of animals. With the presence of penicillin that targets the PBPs and weakens the cell wall of *S. pneumoniae*, the peptide molecule can now act much rapidly and directly to the cell wall and other cellular components thus reducing the time and probably the number of peptide monomers required per pneumococcal cell to initiate killing. Finally, the therapeutic effects of DM3 can be expressed more efficiently. Thus, the specific antimicrobial actions of DM3 on *S. pneumoniae* as well as other bacteria will need further investigations to allow better understanding of the mechanism of actions of the peptides.

CHAPTER 5

CONCLUSION

The escalating reports of antibiotic resistance *S. pneumoniae* has prompted for the search for better disease prevention and treatment strategies in the course of pneumococcal infections. Those at the extreme age groups represent the most commonly infected groups by pneumococcal diseases and vaccinations against *S. pneumoniae* represent the most important prevention strategy. The 23-valent pneumococcal polysaccharide vaccine was designed for the elderly adults and various generations of pneumococcal conjugate vaccines (PCV7, PCV10, PCV13) have been developed for the younger children. However, these pneumococcal vaccines are essentially serotype-specific and thus continued surveillance is needed to predict the effectiveness of vaccines among the local population. Such information is very limited in Malaysia. Our single-center surveillance study based in the densely-populated area of Selangor showed that the prevailing serotypes belonged to the PCV7 vaccine-serotypes. Thus, the PCV7 vaccine is predicted to cover 64.2% of the total cases. In addition, the proportion of penicillin nonsusceptible isolates remains high which constituted half of the cases. Notably, the PCV7 vaccine also covers 87.5%, 79.5%, and 45.3% of the PRSP, PISP, and PSSP, respectively. Hence, the use of PCV7 vaccine and the newer generations of PCVs would benefit the Malaysian population.

The widespread use of conventional antibiotics for the past decades has led to the selection of antibiotic resistant *S. pneumoniae* which complicates empirical therapy and patient management. Newer antimicrobial agents are increasingly demanded for the treatment against this pathogen which has developed substantial tolerability to the conventional antibiotics. We have generated a total of five designed AMPs (DM1, DM2, DM3, DM4, and DM5) which showed potent *in vitro* antipneumococcal activity irrespective of the penicillin susceptibility of the pneumococcal isolates. The pneumocidal kinetics were clearly higher than penicillin and the treated pneumococci were observed with extensive damages especially the cell wall and plasma membrane

which leads to cell death. These peptides were broad spectrum against various gram-positive and gram-negative bacteria that frequently cause life-threatening infections in patients. Among the five hybrids, DM3 was the most potent antimicrobial peptide.

From the therapeutic efficacy testing in mice lethally infected by a PRSP strain, DM3 at 40 mg/kg protected half (50%) of the mice from deaths. The mice showed no apparent sign or symptoms of infections. Notably, the combination treatments with penicillin greatly enhanced the therapeutic outcome of the infected mice in a synergistic manner. This synergism was also seen in *in vitro* assays. Almost all (90%) of protection was achieved with the low doses combination of DM3 at 20 mg/kg with penicillin at 10 mg/kg. Using both agents at 20 mg/kg ensured all (100%) mice survived in the otherwise lethally infected mice.

DM3 showed great potential to be further developed into a clinically useful therapeutic drug whether as a standalone agent or in combination therapy with penicillin to support the conventional antibiotics to improve treatment outcomes especially involving antibiotic resistance strains of pneumococci.

From the current study, the antimicrobial activity and toxicity of DM3 have been investigated using various *in vitro* and *in vivo* procedures. Even so, the actual mechanism of actions of DM3 in its antipneumococcal activity remains largely unclear. The computational simulation would be a valuable technique towards predicting the docking of peptide onto the specific potential target that eventually leads to the killing of *S. pneumoniae*. In addition, evaluating the pharmacokinetic/pharmacodynamic aspects of the peptide will be as important to study the various changes and interactions that take place upon administered into the body of animal as well as human. These information are crucial and shall form the basis in our future explorations towards developing DM3 as a potential novel antibiotics.

In conclusion, antimicrobial peptides represent the most promising class of antimicrobial agents for the development of novel antibiotics. Its simplicity in the primary amino acid sequence and the secondary structure would allow the design of new AMPs with less computational burden. With the rapid expansion in the understanding and strategies in designing of peptides, AMPs could be the next-generation antibiotics with potent antimicrobial activity and low toxicity. We further emphasized the importance of continuous surveillance in order to better understand the local pneumococcal serotype and antibiotic susceptibility patterns.

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APPENDICES

APPENDIX 1: PREPARATION OF CULTURE MEDIA, REAGENTS, AND CHEMICALS

Gram staining reagents

1) Crystal violet
    a. Solution 1; Crystal violet (2 g), ethanol 95% (20 ml)
    b. Solution 2: Ammonium oxalate (0.8 g), distilled water (80 ml)
    c. Solution 1 and 2 were mixed and allowed to stand for at least 24 hrs, and filtered before use.

2) Lugol's iodine
    a. Potassium iodide (2 g)
    b. Iodine (1 g)
    c. Distilled water (300 ml)

3) Diluted carbol fuschin
    a. Stock solution: Safranin O (2.5 g), ethanol 95% (100 ml)
    b. Working solution: Stock solution (10 ml), distilled water (90 ml)

Standard biochemical reagents

1) Bile reagent:
    a. Sodium deoxycholate (0.2 g)
    b. Distilled water (10 ml)

2) Catalase reagent:
    a. Hydrogen peroxide (3%)
    b. Store in refrigerator

3) Phosphate buffered saline
    a. 1 PBS table/100ml distilled water
    b. Autoclave sterilized

4) Tris-EDTA (TE) buffer:
    a. 1 M Tris-HCl, pH 8.0 (1ml, final concentration of 10 mM)
    b. 0.5 M EDTA (200 µl, final concentration of 1 mM)
    c. Distilled water (100 ml)
    d. Autoclave sterilized.

5) Tris-acetate EDTA (TAE) buffer (50X)
    a. Tris base (242 g)
    b. Glacial acetic acid (57.1 ml)
    c. EDTA (18.61 g)
    d. Distilled water (1000 ml)
    e. Autoclave sterilized.

6) Electrophoresis 2% agarose gel
    a. Agarose powder (1.5g)
    b. Distilled water (100 ml)
    c. Microwave boiled until all powder completely dissolved.

7) Magnesium stock solution (10 mg Mg^{2+} /ml)
    a. Powder magnesium chloride hexahydrate, $MgCl_2 . 6H_2O$ (8.36 g)
    b. Deionized distilled water (100 ml)
    c. The powder was dissolved completely and filter sterilized using 0.2 µm syringe filter membrane. Stored at 2 ^oC – 8 ^oC.

8) Calcium stock solution (10 mg Ca^{2+} /ml)
    a. Powder calcium chloride dihydrate, $CaCl_2 . 2H_2O$ (3.68 g)
    b. Deionized distilled water (100 ml)
    c. The powder was dissolved completely and filter sterilized using 0.2 µm syringe filter membrane. Stored at 2 ^oC – 8 ^oC.
    d. The powder was dissolved completely and filter sterilized using 0.2 µm syringe filter membrane. Stored at 2 ^oC – 8 ^oC.

9) Proteinase K solution (10 mg/ml)
    a. Proteinase K powder (25 mg)
    b. Distiller water (2.5 ml)
    c. Dissolved completely. Stored at -20^oC.

10) Lysostaphin solution (50 mg/ml)
    a. Lysostaphin powder (25 mg)
    b. Distilled water (0.5 ml)
    c. Dissolved completely. Stored at -20^oC.

11) 10% buffered formalin
    a. PBS tablet (10 tablets)
    b. 40% formalin (R & M chemicals, UK) (100 ml)
    c. Distilled water (900 ml)
    d. PBS tablets were dissolved in distilled water followed by addition of 100 ml 40% formalin to make 10% buffered formalin.

Bacterial culture agar/broth

1) Blood agar
    a. Tryptone/peptic digest of animal tissue (10 g)
    b. Brain heart infusion (500 g)
    c. Sodium chloride (5 g)
    d. Agar (15 g)
    e. Distilled water (950 ml)

f. Autoclave sterilized. Media was cooled to 50^{o}C – 55^{o}C and 50 ml of preheated (45^{o}C) defibrinated sheep blood was added before agar pouring.

2) Mueller Hinton Agar:
    a. Beef infusion (300 g)
    b. Starch (1.5 g)
    c. Casein (17.5 g)
    d. Agar (17 g)
    e. Distilled water (1000 ml)
    f. Autoclave sterilized. Media was cooled to 50^{o}C – 55^{o}C before agar pouring. For blood supplement 50 ml of preheated (45^{o}C) defibrinated sheep blood may be added.

3) Mueller Hinton broth:
    a. Beef infusion (300 g)
    b. Starch (1.5 g)
    c. Casein (17.5 g)
    d. Distilled water (1000 ml)
    e. Autoclave sterilized.

4) Cationically-adjusted Mueller Hinton broth (CAMHB)
    a. Mueller Hinton broth (997.32 ml)
    b. Chilled calcium stock solution (2 ml)
    c. Chilled magnesium stock solution (0.68 ml)
    d. Both calcium and magnesium stock solutions were added to Mueller Hinton broth mixed well.

5) Nutrient agar:
    a. Nutrient agar (Oxoid, UK) (28 g)
    b. Distilled water (1000 ml).
    c. Autoclave sterilized.

6) Brain Heart Infusion broth:
    a. Calf brain infusion (12.5 g)
    b. Beef heart infusion (5 g)
    c. Glucose (2 g)
    d. Proteose peptone (10 g)
    e. Sodium chloride (5 g)
    f. Disodium phosphate (2.5g)
    g. Distilled water (1000 ml)
    d. Autoclave sterilized.

7) Bacteria stocking medium
    e. Brain heart infusion broth (90 ml)
    f. Glycerol (10 ml)
    g. Autoclave sterilized.

Cell culture media and reagents

1) RPMI-1640 medium (serum free)
    a. RPMI-1640 (supplemented with L-glutamine) in powder form (Flowlab, Australia)
    b. Sodium bicarbonate (3.7 g)
    c. Distilled water (1000 ml)
    d. Filtered sterilized.

2) RPMI-1640 growth medium (with 10% fetal bovine serum)
    a. Serum free RPMI-1640 medium (900 ml)
    b. Fetal bovine serum (heat-inactivated) (100 ml)

3) RPMI-1640 maintenance medium (with 2% fetal bovine serum)
    a. Serum free RPMI-1640 medium (980 ml)
    b. Fetal bovine serum (heat-inactivated) (20 ml)

4) Ham's F12 medium (serum free)
    a. Liquid Ham's F12 medium (Hyclone)

5) Ham's F12 medium growth medium
    a. Liquid Ham's F12 medium (Hyclone) (900 ml)
    b. Fetal bovine serum (heat-inactivated) (100 ml)

6) Ham's F12 medium maintenance medium
    a. Liquid Ham's F12 medium (Hyclone) (980 ml)
    b. Fetal bovine serum (heat-inactivated) (20 ml)

7) Cell line stocking medium
    a. Serum free RPMI-1640/Ham's F12 (24 ml)
    b. Fetal bovine serum (12 ml)
    c. Dimethyl sulfoxide (4 ml)

APPENDIX 2: A REPRESENTATIVE PEPTIDE ANALYSIS REPORT

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CERTIFICATE OF ANALYSIS

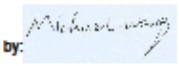
Product Name	DM3
Order ID	168565_8
Lot No.	168565008041812LC09
Sequence	GLFDIWKWWRWRR
Modification	Amidation (C-Terminal);
Length	13AA
Storage	-20°C
Recommended Solvent*	Aqua, pH7.0

Test Items	Specifications	Results
Molecular Weight	Theoretical MW:1904.24	Consistent
HPLC purity	>95%	96.2%
Appearance	White lyophilized powder	Conforms
Quantity	300 mg	300 mg

*Note: This recommendation is based on peptide sequence analysis. For more precise dissolving conditions, please select our peptide solubility test service with an added fee.

Caution:

For laboratory or further manufacturing use only. Not intended for household use. If you have any questions about the Certificate of Analysis, please contact our customer service representative at 1-877-436-7274 (Toll-Free), or 1-732-885-9188.

Certified by:  Date: 2012-05-01

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APPENDIX 2 (CONTINUED)

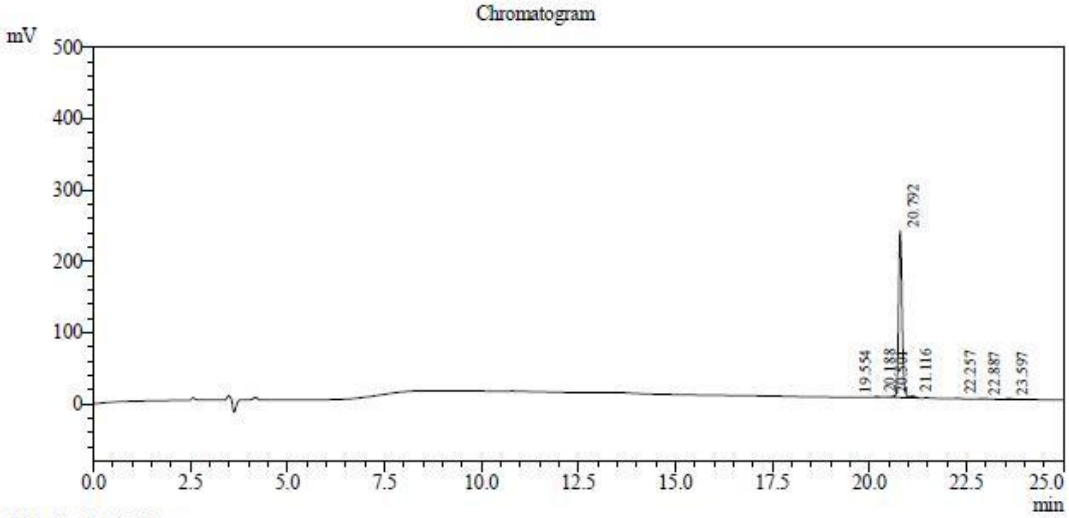
Sample Name : DM3
Sample ID : 168565-8

Pump A : 0.065% trifluoroacetic in 100% water (v/v)
Pump B : 0.05% trifluoroacetic in 100% acetonitrile (v/v)
Total Flow:1 ml/min
Wavelength:220 nm

Time	Unit	Command	Value	Comment
0.01	Pumps	Pump A B.Conc	5	
25.00	Pumps	Pump A B.Conc	65	
25.01	Pumps	Pump A B.Conc	95	
31.00	Pumps	Pump A B.Conc	95	
31.01	Pumps	Pump A B.Conc	5	
40.00	Pumps	Pump A B.Conc	5	
40.01	Controller	Stop		

<<Column Performance>>
<Detector A>
Column : AlltimaTM C18 4.6 x 250 mm

Chromatogram



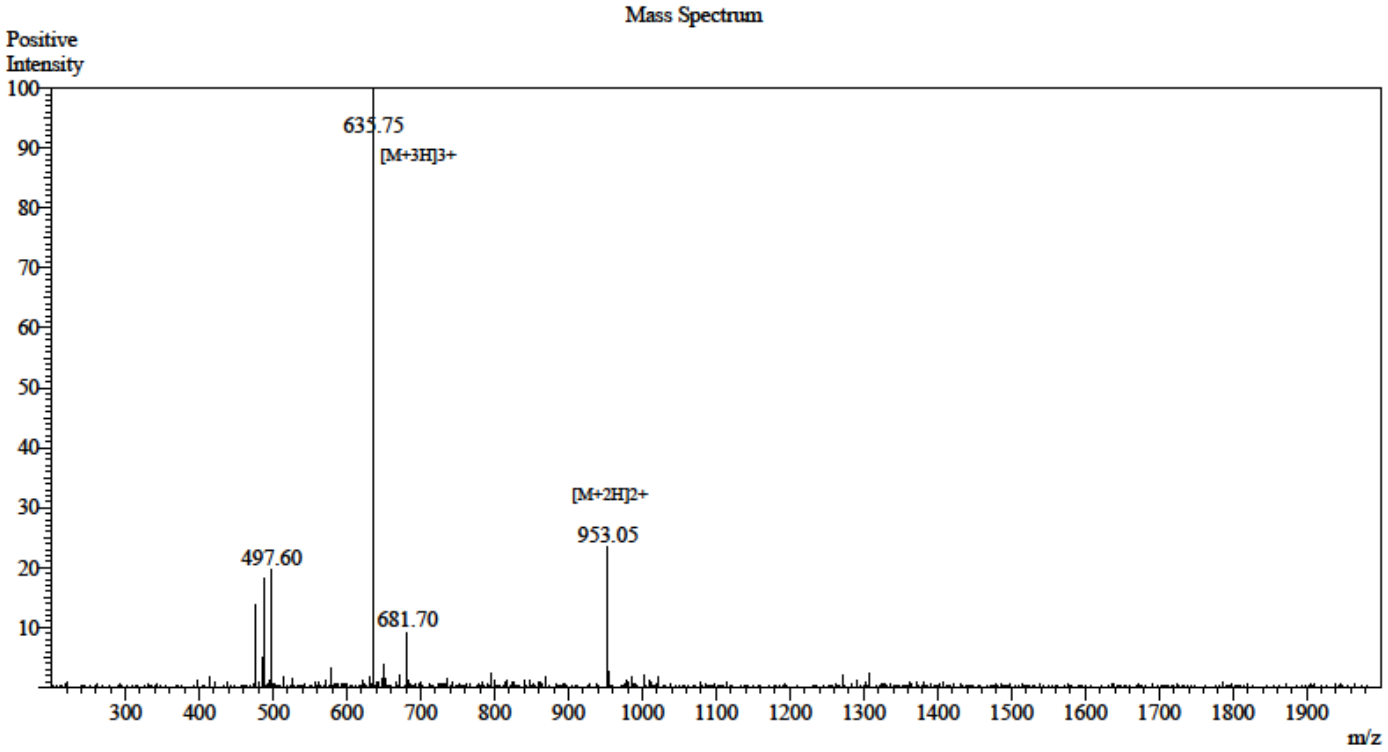
1 Det.A Ch1 / 220nm

Peak Table

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	19.554	2781	463	0.182
2	20.188	9282	1362	0.608
3	20.501	3865	669	0.253
4	20.792	1469118	233683	96.167
5	21.116	24283	2566	1.590
6	22.257	3921	508	0.257
7	22.887	6498	565	0.425
8	23.597	7920	1153	0.518
Total		1527667	240969	100.000

APPENDIX 2 (CONTINUED)



Sample Information

Acquired by :	Gary
Month-Day Processed :	04/28/12
Time Processed :	10:59:15
Injection Volume :	0.2
Sample Name :	DM3
Sample ID :	168565-8
Theoretical MW :	1904.24
Observed MW :	1904.10

Interface	:ESI
Nebulizing Gas Flow	:1.5L/min
CDL Temp	:250℃
Block Temp	:200℃

Interface Bias	:+4.5kV
Detector	:-1.10kV
T.Flow	:0.2ml/min
B.conc	:50%H2O/50%MeOH

APPENDIX 3: PUBLICATIONS AND PRESENTATIONS

Publications

1) **Le, C. F.**, Jefferies, J. M., Yusof, M. Y., Sekaran, S. D., & Clarke, S. C. (2012). The epidemiology of pneumococcal carriage and infections in Malaysia. *Expert Rev Anti Infect Ther, 10*(6), 707-719. doi: 10.1586/eri.12.54

2) **Le, C. F.**, Mohd Yusof, M. Y., & Sekaran, S. D. (2011). Current trend in pneumococcal serotype distribution in Asia. *J Vaccines Vaccin*(Pneumococcal Vaccination), S2:001. doi: 10.4172/2157-7560.S2-001

3) **Le, C. F.**, Palanisamy, N. K., Mohd Yusof, M. Y., & Sekaran, S. D. (2011). Capsular serotype and antibiotic resistance of Streptococcus pneumoniae isolates in Malaysia. *PLoS One, 6*(5), e19547. doi: 10.1371/journal.pone.0019547

4) Palanisamy, N. K., Mohd Yusof, M. Y., Ong, S. Y., Mansor, M., **Le, C. F.**, & Sekaran, S. D. (2009). Variation of sequence of genes encoding the MurMN Operon and cell wall composition in Streptococcus pneumoniae strains of different susceptibility levels to penicillin. *J Infect Dis Antimicrob Agents, 26*(3), 12.

5) **Le, C. F.**, Mohd Yusof, M. Y., & Sekaran, S. D. Redesigning novel synthetic antimicrobial peptides with improved therpaeutic efficacy against *Streptococcus pneumoniae*. (Manuscript in preparation)

Oral presentations:

1) **CF Le**, MA Abdulla Hassan, MY Md Yusof, SD Sekaran. DM3, A Novel synthetic antimicrobial peptide with potent *in vitro* and *in vivo* antimicrobial activities against *Streptococcus pneumoniae*.
   Presented at the 9th International Symposium on Antimicrobial Agents and Resistance (ISAAR 2013), 13th – 15th March 2013, Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia (also presented as poster presentation).
   ***Young Investigator Award**

Poster presentations:

1) **CF Le**, MY Md Yusof, SD Sekaran. Pneumococcal serotype distribution in Malaysia.
   Presented at the 9th International Symposium on Antimicrobial Agents and Resistance (ISAAR 2013), 13th – 15th March 2013, Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia.

2) **Le Cheng Foh**, Mohd Yasim Md Yusof, and Shamala Devi Sekaran. Novel synthetic antimicrobial peptides with potent *in vitro* and *in vivo* antimicrobial activities against *Streptococcus pneumoniae*.
   Presented at the Faculty of Medicine Research Week, 21st – 23rd January 2013, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia.

3) Shamala Devi Sekaran, Mohd Yasim Md Yusof, **Le Cheng Foh**. DM3, a novel designed antimicrobial peptides protects mice from lethal pneumococcal infections.
   Presented at the BioMalaysia 2012, 5th – 7th November 2012, Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia.
   ***BioInnovation Award, Bronze medal**

4) Shamala Devi Sekaran, **Le Cheng Foh**, Alwin Kumar Rathinam, Mohd Yasim Md Yusof. New self-designed drug for treatment of *Streptococcus pneumoniae* infections.
   Presented at the BioMalaysia 2011, 21st – 23rd, November 2012, Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia.
   ***BioInnovation Award, Bronze medal**

5) **Le Cheng Foh**, Alwin Kumar Rathinam, Mohd Yasim bin Mohd Yusof, Shamala Devi Sekaran. Synthetic antimicrobial peptide as alternative to antibiotic for the treatment of *Streptococcus pneumoniae* infections

   Presented at the University of Malaya Innovation and Creativity Expo 2010 (UMEXPO 2010), 1st – 3rd April 2010, University of Malaya, Kuala Lumpur, Malaysia.

   ***Silver Medal**

6) **Le Cheng Foh**, Mohd Yasim Yusof, and Shamala Devi Sekaran. Identification of novel antibiotic resistance targets and development of antimicrobial peptides to *Streptococcus pneumoniae*.

   Presented at the International Congress of Malaysian Society for Microbiology (ICMSM 2009), 1st – 4th December 2009, Park Royal Hotel Penang, Penang, Malaysia.

Awards and Recognitions

1) **Young Investigator Award –** 9th ISAAR 2013, Kuala Lumpur , Malaysia
2) **BioInnovation Award, Bronze medal** – BioMalaysia 2012, Kuala Lumpur, Malaysia
3) **BioInnovation Award, Bronze medal** – BioMalaysia 2011,, Kuala Lumpur, Malaysia
4) **Silver medal –** 10th UMEXPO, University of Malaya, Kuala Lumpur, Malaysia
5) **Full sponsorship –** Introductory Bioinformatics Workshop, 9th – 14th July 2012, Perdana University, Selangor, Malaysia
6) **Full sponsorship to the School of Medicine, University of Southampton, UK** – 1 month international student training under the supervision of Dr. Stuart C. Clarke, University of Southampton School of Medicine, Southampton, UK.
7) **University of Malaya Scholarship Scheme (SBUM)** – 1st July 2009 – 31st March 2013
8) **Postgraduate research Fund** – PS187/2009B
9) **Postgraduate research Fund** – PV077/2011B

Oral presentation - Young Investigator Award

Presented at the 9th International Symposium on Antimicrobial Agents and Resistance (ISAAR 2013), 13th – 15th Mac 2013, Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia.

DM3, A Novel synthetic antimicrobial peptides with potent *in vitro* and *in vivo* antimicrobial activities against *Streptococcus pneumoniae*

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Background: *Streptococcus pneumoniae* is a major human bacterial pathogen causing significant morbidity and mortality worldwide. The increasing reports of antibiotic-resistant pneumococci have prompted the search for novel antibiotics. Methods: Five synthetic antimicrobial peptides (DM1-5) were designed and tested by standard methods such as minimum inhibitory concentration (MIC), killing kinetics, transmission electron microscopy (TEM), hemotoxicity, and cell cytotoxicity against lung cell lines. *In vivo* acute toxicity and therapeutic efficacy was evaluated using an in-house mouse pneumococcal infection model. Results: The five DMs showed potent antipneumococcal activities against 60 pneumococcal isolates irrespective of penicillin resistance of the isolates (MIC = 7.81- 250μg/ml) with broad spectrum activity against multiple bacterial pathogens. Pneumococcal killing kinetics was higher than penicillin by 30 – 75% and the DMs induced overwhelming cellular damages leading to pneumococcal cell death. Hemolytic activity was minimal (HC_{50} > 250 μg/ml) while IC_{50} against lung cell lines ranged from 96 - >250μg/ml. Intraperitoneal treatment with DM3 (40mg/kg) protected 50% of mice from a lethal pneumococcal systemic infection model challenged by a penicillin-resistant strain. Combinations of DM3 and penicillin produced therapeutic synergism by enhancing the survival rates to 90% (DM3 20mg/kg, PEN 10mg/kg) and 100% (DM3 20mg/kg, PEN 20mg/kg). Conclusion: DM3 showed great potential as a standalone antimicrobial candidate or in formulation with conventional antibiotics to enhance treatment outcome in pneumococcal infections especially cases involving resistant pneumococci.

All authors have no conflicts of interest with regard to this presentation

Poster presentation 1

Presented at the 9th International Symposium on Antimicrobial Agents and Resistance (ISAAR 2013), 13th – 15th March 2013, Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia.

PNEUMOCOCCAL SEROTYPE DISTRIBUTION IN MALAYSIA

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Background: *S. pneumoniae* causes a significant proportion of pneumonia and meningitis in Malaysia. Pneumococcal-conjugate vaccines (PCVs) have been the most promising immunization strategy to the younger children. However, PCVs are serotype-specific and local serotype distribution is important to evaluate the potential coverage of PCVs. Methods: Multiplex serotyping was performed on 208 pneumococcal isolates obtained from the microbiology laboratory of University of Malaya Medical Centre, Kuala Lumpur from 1997 – 2012 (except 2001 and 2004). Minimum inhibitory concentration (MIC) was determined using agar dilution method (isolates obtained on 2007 and before) and broth microdilution method (isolates obtained after year 2007). Grouping of penicillin susceptibility followed the penicillin (oral). Statistical analysis was performed using chi-squared or fisher's exact tests whenever appropriate.Results: Serotype 19F represented the major serotype (33%), followed by 23F (9%), 6A/B (6%), 1 (6%), and 14 (5%). Half of the total isolates (51%) were penicillin non susceptible (PNSP). Serotypes were found to highly associated with penicillin susceptibility ($p<0.001$) with serotype 19F being the most resistant serotype (62%). In fact, 78% of serotype 19F was PNSP. Most isolates (30%) were obtained from nasopharyngeal (NP), followed by sputum (26%) and blood (24%). Statistical test showed that serotype 19A was associated with invasive site (11%) while 19F was associated with non invasive site (44%). Interestingly, the invasive isolates were likely to be penicillin susceptible while non invasive isolates were likely to be PNSP. Conclusion: PCV7, PCV10, and PCV13 are expected to cover 56%, 63%, and 71% of Malaysian serotypes. The use of PCVs in Malaysia is still limited and incorporation of PCVs under the childhood immunization scheme is encouraged.

Poster presentation 2

Presented at the Faculty of Medicine Research Week, 21st – 23rd January 2013, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia.

Novel synthetic antimicrobial peptides with potent *in vitro* and *in vivo* antimicrobial activities against *Streptococcus pneumoniae*

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Abstract

Streptococcus pneumoniae is a major human bacterial pathogen causing significant morbidity and mortality worldwide. The increasing reports of antibiotic-resistant pneumococci have prompted the imperative search for novel antimicrobial agents. We have generated a series of synthetic antimicrobial peptides and found that five peptides (DM1-5) showed potent antipneumococcal activities and rapid killing rates irrespective of the penicillin resistance of the pneumococcal strains. These peptides induced overwhelming pneumococcal cell wall and cell membrane damages which eventually lead to cell death as observed using Transmission Electron Microscopy. The DMs were able to produce synergism in combination with the conventional antibiotic penicillin. Interestingly, DMs also displayed broad spectrum antibacterial activity against other common clinically-encountered bacterial pathogens such as *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, and others. The hemolytic activities were low while cytotoxicities against two human lung cell lines varied among the DMs. Further testing of these peptides in mouse infection model found that intraperitoneal treatment with DM3 (40mg/kg) for three doses at 2hrs, 12hrs, and 24hrs protected 50% of mice from lethal pneumococcal infection by a penicillin-resistant strain. Mice treated with this DM3 regimen have no major abnormalities in blood haematogram and serum biochemistry parameters as well as histopathology of five major organs (spleen, liver, lung, kidney, brain). Interestingly, combination treatments using DM3 and penicillin produced *in vivo* therapeutic synergism. While DM3 (20mg/kg) and penicillin (20mg/kg) conferred 20% and 50% protection to the mice, all mice (100%) treated with this DM3-penicillin combination survived from the lethal pneumococcal challenge. Antimicrobial peptides represent the promising novel antimicrobial agent and here we have developed DM3 showing great potential as antimicrobial candidate in standalone form or to be formulated in combination with conventional antibiotics to enhance pneumococcal infections treatment outcome especially those involving pneumococcal-resistant strains.

Poster presentation 3 – BioInnovation Award, Bronze Medal

Presented at the BioMalaysia 2012, 5th – 7th November 2012, Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia.

DM3, a novel designed antimicrobial peptides protects mice from lethal pneumococcal infections.

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Description of innovation

Streptococcus pneumoniae is responsible for life-threatening infections including meningitis, bacteremia, and pneumonia affecting primarily children < 2 years of age and the elderly adults. Treatment against pneumococcal infections depends mainly on beta-lactam antibiotics such as penicillin and cephalosporins. Nevertheless, increasing reports of antibiotic-resistant *S. pneumoniae* have greatly restricted the choice of antibiotics as well as the treatment outcome. Novel antimicrobial agents are urgently needed as the alternatives therapeutic choices.

We have designed a novel peptide-based antimicrobial drug, DM3 which is completely different from the conventional antibiotics in terms of the chemical structure and the antibacterial properties. DM3 displayed potent antimicrobial activity against *S. pneumoniae* including the penicillin-resistant strains. DM3 is also broad spectrum against multiple common clinically encountered bacteria including *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Pseudomonas aerugenosa*, *Escherichiae coli*, *Acinetobacter baumanii*, *Enterococcus cloacae*, and *Citrobacter* spp.. These have been experimentally proven in our laboratory using various antimicrobial testing methods. DM3 eliminated >95% pneumococcal cells almost instantaneously post treatment and the activity was superior to penicillin by >55%. DM3 synergized and enhanced the antipneumococcal potency of penicillin when both are given in combination. DM3 is considerable non-toxic in that toxicity was only seen at 125μg/ml which was two-fold higher than the MIC range. In *in vivo* experiment, DM3 protected a significant portion of mice infected with a highly lethal strain of *S. pneumoniae* with no manifestation of clinical illnesses post treatment. Thus, continued development of DM3 is envisioned to provide an alternative choice in treatment of bacterial infections whether as standalone or to support the conventional treatment regimen especially in cases involving antibiotic-resistant strains. This type of peptide-based antibiotics is novel to the pharmaceutical industry and hence indicates that the potential for this new generation antibiotics is exceptionally great.

Poster Presentation 4 - BioInnovation Award, Bronze Medal

Presented at the BioMalaysia 2011, 21st – 23rd, November 2012, Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia.

New self-designed drug for treatment of *Streptococcus pneumoniae* infections.

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Description of innovation

Streptococcus pneumoniae is one of the major human bacterial pathogens causing life threatening diseases such as meningitis, bacteremia, and pneumonia. Children <2 years of age and the elderly adults are the most heavily infected groups. Treatment against pneumococcal infections depends mainly on beta-lactam antibiotics such as penicillin and cephalosporins. Nevertheless, increasing reports of antibiotic-resistant *S. pneumoniae* have greatly restricted the choice of antibiotics as well as the treatment outcome. Hence, the search for new generation antimicrobial agents is urgently needed.

Current innovation described the development of three novel peptide-based antibiotics, strepDM1, strepDM4, strepDM5. The peptides are different from and not the synthetic variants of the conventional antibiotics and hence the inherent resistances exhibited by the pneumococci are not readily expressed against our peptides. These have been experimentally proven *in vitro* in our laboratory using the broth microdilution assay. In addition, the individual peptide cleared >60% of pneumococcal load within 30 minutes posttreatment by just single dose use and the killing persists thereafter and cleared ≥99% of cells in 90 – 180 minutes. Furthermore, our peptides also exhibited broad-spectrum antibacterial activity against diverse bacterial pathogens of both gram types, including *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Pseudomonas aerugenosa, Escherichiae coli, Acinetobacter baumanii, Enterococcus cloacae*, and *Citrobacter* spp. Most importantly, the peptides exhibited no hemotoxicity at concentration equal to or higher than the bacterial inhibitory concentrations.

We envision that our innovation, strepDM could provide alternative choices in treatment of bacterial infections especially cases involving antibiotic-resistant *S. pneumoniae*. The excellent toxicity profile allows wider choice both in the selection of route and regimens of administrations. Although our peptides are still in early stage of development, they have the potential to be further developed as supporting agents with conventional antibiotics marketed or as standalone therapeutic agents.

Poster presentation 5 – Silver Medal

Presented at the University of Malaya Innovation and Creativity Expo 2010 (UMEXPO 2010), 1st – 3rd April 2010, University of Malaya, Kuala Lumpur, Malaysia.

Synthetic antimicrobial peptide as alternative to antibiotic for the treatment of *Streptococcus pneumoniae* infections

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Description

Streptococcus pneumoniae is one of major human pathogens, causing various life-threatening diseases such as meningitis, sepsis, pneumonia, otitis media, and others. Although penicillin remain as principal drug for treating pneumococcal infections, the increase emergence of penicillin-resistant *Streptococcus pneumoniae* will eventually render the antibiotic ineffective. Therefore, there is an urgent need in search of novel therapeutics against the bacteria.

The natural antimicrobial peptides (NAMPs) are increasingly recognized as important components constituting the first line of defense in mammalian innate immunity. They are generally short and rapid acting polycationic antimicrobial peptides exhibit broad spectrum antimicrobial activities against diverse array of bacteria, fungi, parasites, and some viruses. They can be found naturally from various organisms from human, mammals, insects, amphibians, and others.

Although many natural peptides have been described, there is great potential for the development of synthetic analogs, based on existing structures, with increased antimicrobial activity. By using various biocomputationl approaches, we have designed a group of synthetic antimicrobial peptides (DAMPs) that targeting the penicillin-binding proteins (PBPs) of *S. pneumoniae*. Two of our designed peptides, DAMP 6 and DAMP 7 found to have substantial antipneumococcal activity, with DAMP 6 having no toxicity although DAMP 7 has observed concentration-dependent toxicity.

Although this product are still at the earlier stage of testing, with future works on the *in vivo* and preclinical evaluation of peptides, the successful development of this products as new therapeutic against *S. penumoniae* will bring about great impact to the community. The direct benefit is seen as improving the health status of the people. Each year at least 1 million children younger than 5 years of age die of pneumonia and invasive diseases in developing countries. Mortality rates for pneumonia and meningitis are especially high for young children, the elderly, and immunocompromised individuals, including patients with human immunodeficiency virus. The demand for antibiotics are always high. For commercialization, these peptides can potentially be marketed as a new drug for *S. pneumoniae,* giving the patients and physicians a new choice of drug. In addition, this can help to reduce the financial burden of individual as well as the government, at which billions of Ringgit can be saved from the country's each year for development of country.

Previous works from other researchers have focused mainly on membrane active peptides, which owes its activity via perturbation of cell membrane leading to cell lysis. However, our design strategy in this study does not utilize the same approach, instead we target the penicillin-binding protein (PBPs). To our knowledge, this strategy is first implicated in drug design from similar field. The advantage over the membrane-

disruption approach is due to the possible cytotoxicity arise as a result of non-specific activity of peptides on both the desirable bacterial membrane targets, as well as the undesirable activity on human cells, especially erythrocytes.

Although the stage of drug development in the current study is still early, but the impact that it can bring about is massive to the field of drug development. Basically, the idea applied is simple: to manipulate the sequences of peptides which are critical to their actions in order to generate new products with altered properties. Similar approaches can be use for design of synthetic peptides against other components of *S. penumoanie* or other pathogens. In addition, the discovery that can be made here is unlimited because we can virtually generate any sequences of peptides of various length and multiple combination of amino acids, not taking account others (N-terminal and C-terminal modifications, D-amino acids, succinylation, amide cyclic, disulfide bridge, and etc).

Poster Presentation 6

Presented at the International Congress of Malaysian Society for Microbiology (ICMSM 2009), 1st – 4th December 2009, Park Royal Hotel Penang, Penang, Malaysia.

Identification of novel antibiotic resistance targets and development of antimicrobial peptides to *Streptococcus pneumoniae*

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Abstract

Streptococcus pneumonia, a gram-positive, alpha-hemolytic aerotolerant diplococcus that present as normal flora in the upper respiratory tracts. It was a major cause of pneumonia in the late 19th century and also causes many other life-threatening diseases. Moreover, the increasing reports of multi-drug resistant *S. pneumoniae* worldwide is no more an issue, it is catastrophic if no alternative therapeutic agents be made available in years time. In this study, we utilize natural antimicrobial peptides (NAMP) as our reference peptides against which the synthetic antimicrobial peptides (DAMP) were designed from, with *S, pneumoniae* penicillin-binding protein as the target site . We constructed a small library of NAMP via screening into several peptide databases. Methods employed in designs and modifications of DAMP include simple amino acid substitution, addition/deletion, and/or short chain hybridization in order to generate DAMP with variable biochemical properties. *In vitro* assays were performed to profile the antimicrobial, cytotoxicity, and hemolytic activities of peptides. Our preliminary MIC results showed promising antipneumococcal activity by several peptides tested. Furthermore, most of the peptides tested are either having no or only with minimum level of hemolytic and cytotoxic activities. The results presented here illustrate that antimicrobial peptides can be generated through construction of small libraries. We hope that the findings from this study could lead to more progressive development of antimicrobial peptides against *S. pneumoniae*.